

PLANT PLASMA MEMBRANES

ISOLATION AND CHARACTERIZATION

Per Kjellbom



Lund 1986

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by

Civ. ing. Per Kjellbom (Sm)

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Abstract The plasma membrane, or plasmalemma, is the differentially permeable barrier that delimits the living plant cell from its external environment. The plasma membrane is therefore bound to have many important functions in relation to the interaction between the cell (i.e., the protoplast) and its surroundings, and it must constantly counteract the forces of equilibrium in order for cells, and thus whole organisms, to live. This thesis presents an aqueous polymer two-phase procedure for the purification of plant plasma membrane vesicles from light-grown barley and spinach leaves. Analysis of marker enzyme activities and specific staining of the plasma membrane indicate a purity close to 100 %. By using low-temperature spectroscopy I present evidence for the presence of a blue-light-reducible b-type cytochrome in the leaf plasma membrane, similar to the b-type cytochrome earlier identified in plasma membranes of dark-grown tissues and inferred to have a role in blue-light photomorphogenesis. I also show that the plasma membrane has a low protein content compared to intracellular membranes. Furthermore, the polypeptide composition of spinach and barley plasma membranes are revealed by SDS-PAGE. The plasma membrane vesicles obtained by phase partition are sealed and right side-out, as shown by measuring the latency of membrane marker enzyme activities upon addition of a detergent. Plasma membrane vesicles prepared from barley roots and leaves have nearly identical polypeptide composition, albeit with different relative amount of some of the polypeptides. The isoelectric points of the outer surface of root and leaf plasma membrane vesicles, as determined by cross-partition, were found to be similar and quite acidic (pH 3.6). In contrast, the partition at the isoelectric point and the surface net charge density at pH 7.0, as determined by 9-aminoacridine fluorescence, differed significantly. Furthermore, the presence of cytochrome P-450/420 in plant plasma membranes is indicated by CO-difference spectra and pyridine binding spectra, and may thus be identical to the blue-light-reducible b-type cytochrome.			
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Signature Per Kjellbom

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Separate publications

List of separate publications

This thesis is based on the following publications, which will be referred to by their Roman numerals.

- I. Preparation and polypeptide composition of chlorophyll-free plasma membranes from leaves of light-grown spinach and barley.
Per Kjellbom and Christer Larsson
Physiol Plant 62: 501-509 (1984)
- II. Sidedness of plant plasma membrane vesicles purified by partitioning in aqueous two-phase systems.
Christer Larsson, Per Kjellbom, Susanne Widell and Tomas Lundborg
FEBS Lett 171: 271-276 (1984)
- III. Surface properties of right side-out plasma membrane vesicles isolated from barley roots and leaves.
Lena E Körner, Per Kjellbom, Christer Larsson and Ian M Møller
Plant Physiol 79: 72-79 (1985)
- IV. Cytochrome P-450/420 in plant plasma membranes: A possible component of the blue-light-reducible flavoprotein-cytochrome complex.
Per Kjellbom, Christer Larsson, Per Askerlund, Cecilia Schelin and Susanne Widell
Photochem Photobiol 42: 779-783 (1985)

1. Introduction

The plasma membrane, or plasmalemma, is the differentially permeable barrier that delimits the living plant cell from its external environment. The plasma membrane is therefore bound to have many important functions in relation to the interaction between the cell (i.e., the protoplast) and its surroundings, and it must constantly counteract the forces of equilibrium in order for cells, and thus whole organisms, to live. Interactions of this kind include such diverse phenomena as transport of ions, photosynthetic products and other solutes into and out of the cell (Wyse 1985), the synthesis and assembly of cell wall material such as for instance cellulose (Langebartels et al. 1981), hormone binding and responses (Hertel et al. 1983), host-pathogen interactions (Walker-Simmons et al. 1984), frost hardiness (Yoshida 1984), blue-light photomorphogenesis (Senger and Briggs 1981), and guard cell responses leading to stomata closure and opening, in that way regulating gas exchange and water economy in higher plants (Hornberg and Weiler 1984, Assman et al. 1985, Shimazaki et al. 1986). However, compared to the considerable knowledge available concerning the isolation, organization and different properties of animal plasma membranes our knowledge concerning plant plasma membranes is quite meagre.

The reason for the relatively limited information available on plant plasma membranes compared to animal plasma membranes is largely due to the difficulties involved in the isolation of sufficient quantities of pure plasma membranes from plants. So far 12 distinct types of cellular membranes have been recognized in higher plants (Palade 1983), and the plasma membrane forms a relatively small proportion of total membrane in the cell, ranging from approximately 1 to 13 %, depending on the kind of material studied (I, Morré and Buckhout 1979). To separate the plasma membrane from intracellular membranes, different density gradient centrifugation techniques have traditionally been used, however with limited success (Hall 1983). The problems involved in using such techniques are especially evident when preparing plasma membranes from green tissue since thylakoid membranes are not easily removed from plasma membranes by separation methods based on differences in density (Tab. 1). In addition to having approximately the same density as plasma membranes, thylakoid membranes are likely to constitute well over 90 % of total membrane in a mesophyll cell of a green leaf.

Some of the events likely to take place at the plasma membrane are probably also restricted to certain organs of the plant. This should be the case for export of carbon assimilation products, an

event restricted to the photosynthesizing leaf cells. Similarly many plant-pathogen interactions are confined to certain organs.

A prerequisite for studying plasma membrane associated events in different parts of fully differentiated plants, is the access to purification methods capable of obtaining pure plasma membranes from different organs and tissues, including green leaves where the presence of thylakoid membranes offers a special challenge.

2. Purification strategies

The successful purification of plant plasma membranes has been hampered by two main problems:

(i) The need to use high shear forces to disrupt the plant material (e.g., cell walls) gives rise to a massive formation of membrane vesicles originating from disrupted organelles. Since the plasma membrane has approximately the same density as several intracellular membranes (Tab. 1), difficulties have arisen when density gradient procedures have been used in purifying plant plasma membranes.

(ii) The lack of reliable markers for the plant plasma membrane has introduced considerable uncertainty concerning the purity of the plasma membrane fractions obtained. As will be seen in the following, these obstacles can be approached in several ways.

Tab. 1. Approximate densities of various plant cell components in sucrose density gradients (adapted from Leonard and Hodges 1980).

Cell component	Density (g cm ⁻³)
Tonoplast	<1.13
Smooth endoplasmic reticulum	1.09-1.12
Golgi membranes	1.12-1.15
Rough endoplasmic reticulum	1.15-1.17
Plasma membrane	1.14-1.18
Broken plastids	1.16-1.18
Mitochondria	1.18-1.20
Nuclear membrane	1.21-1.23
Intact plastids	1.21-1.24
Microbodies	1.19-1.27

The first step when purifying plant plasma membranes is usually a homogenization of the plant tissue. The homogenization should be as gentle as possible in order to minimize fragmentation of intracellular structures, but at the same time it must facilitate the separation of the plasma membrane

from the cell wall, with which the membrane is closely associated, e.g., through plasmodesmata (Hall 1983). The composition of the homogenization medium varies widely, presumably reflecting individual experience of different plant material. In most cases an isotonic or slightly hypertonic environment is maintained during homogenization, and media containing 0.25 to 0.50 M sucrose are often used. The homogenization medium is buffered, using for instance 25 to 100 mM Hepes, and kept somewhat above neutral pH, in order to counteract acidic substances released from the vacuoles upon disruption. Other constituents are often present in the homogenization medium. EDTA (0.1-5 mM) is added to chelate divalent cations, thereby reducing phospholipase activity, leading to lower levels of free fatty acids and to a better preservation of membrane structure. EDTA also causes the conversion of rough endoplasmic reiculum (ER) to smooth ER, thereby facilitating a more complete separation of ER from the plasma membranes when the separation is based on differences in density (Tab. 1). Reducing agents and antioxidants, such as dithiothreitol, cysteine and ascorbic acid, are used in order to keep SH-groups reduced and inhibit the activities of oxidative enzymes. Polyvinylpolypyrrolidone or similar compounds may be included to remove phenolic compounds released from the vacuoles.

Upon cell disruption plasma membrane fragments vesiculate because it is thermodynamically unfavourable to expose hydrophobic membrane edges to an aqueous environment (Tanford 1980). The vesicles formed are usually 0.1 to 2.0 μm in diameter, depending upon plant species and homogenization procedure (I, Leonard and Hodges 1980). The material recovered after homogenization is filtered to remove large cell fragments, and most of the chloroplasts and mitochondria are pelleted by low speed centrifugation (e.g., 10 000 g for 10 minutes) and discarded. A microsomal pellet is obtained from the supernatant by ultracentrifugation (30 000-100 000 g for 30 to 60 minutes), and used as starting material for plasma membrane purification.

2.1. Gradient centrifugation

Density gradient centrifugation techniques take advantage of the different densities of membrane vesicles in the gradient material used. Most often variations of the sucrose density gradient centrifugation method of Hodges and Leonard (1974) are utilized when purifying plant plasma membranes.

The buoyant density of a membrane vesicle in a density gradient is dependent on its relative content of protein ($d \approx 1.35 \text{ g cm}^{-3}$), lipids ($d \approx 1.0 \text{ g cm}^{-3}$), polysaccharides ($d \approx 1.6 \text{ g cm}^{-3}$) and water ($d \approx 1.0 \text{ g cm}^{-3}$) (Hinton 1976). The gradient material chosen will determine to what extent water influences the density of membrane vesicles, because water contributes to the density of the membrane only when it is inaccessible to the gradient material. Sucrose, for instance, is much more penetrating than are the polymers Ficoll and Dextran, and deprives membrane vesicles of water to a larger extent. This means that membrane vesicles in sucrose density gradients equilibrate at much higher densities than in gradients of Ficoll or Dextran. Sucrose also exerts a considerable osmotic effect on vesicles, turning vesicles impermeable to sucrose into collapsed structures, and permeable vesicles to spheres with the same sucrose concentration on both sides of the membrane. This is not as pronounced with Ficoll and Dextran, which exert lower osmotic effects. The influence of the gradient material is important to have in mind when visualizing what happens to vesicles passing through a density gradient.

If one wants to isolate a biological membrane by sucrose density gradient centrifugation, one usually begins running a continuous, linear gradient (Hodges and Leonard 1974). According to the distribution of different marker activities in the continuous gradient, a discontinuous density gradient is prepared, in such a way that the membrane that is to be isolated ends up at an interface between two sucrose concentrations with as little contamination by other membranes as possible. Separations based on density gradient centrifugations will solely separate membrane vesicles according to differences in density irrespective of, e.g., what sidedness they possess.

2.2. Protoplasts

By using protoplasts as starting material for plasma membrane preparations the high shear forces needed for tissue homogenization can be avoided and a high proportion of intact organelles can be released by a mild disruption of the protoplasts, thereby facilitating the initial removal of large intact organelles, as chloroplasts and mitochondria, by low-spin centrifugation (Perlin and Spanswick 1980). Protoplast disruption can be obtained by osmotic shock (Wagner 1985), or by forcing the protoplasts through a nylon net (Rathnam and Edwards 1976, Hampp and Goller

1983), or through a long thin needle (Martinoia et al. 1981). Besides density gradient centrifugation, free-flow electrophoresis, and two-phase partition (the two latter techniques will be discussed below), there are several alternative ways of separating released organelles from plasma membranes after disruption of protoplasts. One possibility is to adsorb the intact protoplasts (and thereby the plasma membrane) on certain resins, such as Cytodex (Bornman and Zachrisson 1982), poly-L-lysine (Jacobson 1980), or lectin-Sepharose (Schroeder et al. 1982, Kaplan et al. 1984). Following gentle lysis and washing, the plasma membranes may be recovered, e.g., by elution with a buffer containing α -methyl-mannoside if Con A-Sepharose is used. Another technique involves coating the protoplasts with a layer of cationic silica microbeads followed by lysis and centrifugation. The heavy microbeads (2.5 g cm^{-3}) facilitate the rapid sedimentation of plasma membrane sheets (Wasserman et al. 1984).

Intact protoplasts have been used for the study of some plasma membrane associated events, such as transport (VerNooy and Lin 1986) and redox activities (Lin 1982). In most cases, however, this approach has been used for labeling of plasma membrane proteins, lipids or carbohydrates (Galbraith and Northcote 1977, Boss and Ruesink 1979, Chadwick and Northcote 1980, Perlin and Spanswick 1980).

Thus, protoplasts and plasma membranes prepared from protoplasts may be quite useful for certain types of plasma membrane studies. This approach must be used with caution, however, since a loss of activities often seems to accompany protoplast preparation. An increase in membrane permeability is reported to be one such side-effect (Taylor and Hall 1976). Thus, a careful monitoring of potentially detrimental effects of the cell-wall-degrading enzymes on the event or activity studied is a mandatory control. When using intact protoplasts and substrates that might be metabolized by cytoplasmic constituents or cell organelles it is of utmost importance that the protoplasts really are intact. A small number of leaky protoplasts may be enough to prevent a correct interpretation of experimental results.

2.3. Free-flow electrophoresis

Free-flow electrophoresis (Hannig and Heidrich 1974), which has been used for separation of cells, cell organelles and membrane subfractions (Menashi et al. 1981, Morré et al. 1983), has recently been used for purifying plant plasma membranes (Barr et al. 1985b, Auderset et al. 1986, Sandelius et al. 1986).

By this technique membrane vesicles are separated according to differences in surface net charge density at the pH chosen. When this method was applied to microsomal fractions of dark-grown soybean hypocotyls and light grown spinach leaves (Sandelius et al. 1986, Auderset et al. 1986), most intracellular membrane vesicles were concentrated in a broad major zone towards the center of the chamber. Two additional minor zones, one towards the anode, the other towards the catode, were also obtained. The membrane vesicles in the most electronegative fraction (i.e., towards the anode) were identified as tonoplast vesicles, and the least electronegative material (i.e., towards the catode) were identified as plasma membrane vesicles. These two fractions contained approximately equal amounts of vesicles as measured by protein content, each comprising ca 7 % of total protein.

2.4. Aqueous polymer two-phase systems

Partition in aqueous polymer two-phase systems was introduced by Albertsson in 1958 as a method for fractionation of membrane vesicles according to their surface properties, i.e., charge and hydrophobic/hydrophilic properties (for a comprehensive treatment of the method, see Albertsson 1986). Mixing almost any pair of water soluble polymers will give rise to a phase system, provided that the concentrations are sufficiently high and the polymers sufficiently dissimilar. The polymers generally used are Dextran T 500 (M_w 500 000) and polyethylene glycol 3350 (M_n 3350). At 4°C these polymers will form two-phase systems at concentrations above ca 5 % of each polymer (the "critical concentration").

The first indication that aqueous Dextran-polyethylene glycol two-phase systems could be used for purifying plasma membranes was obtained when subfractionating crude spinach chloroplast

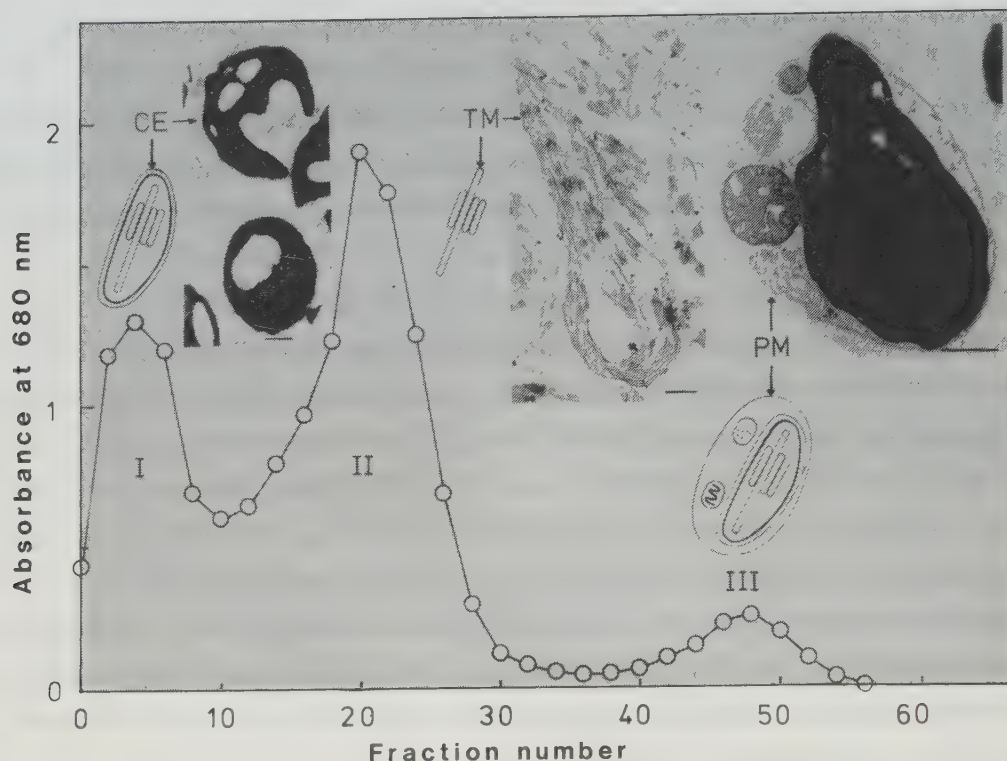


Fig.1. Countercurrent distribution of a crude spinach chloroplast preparation. The peaks contain intact chloroplasts (I), thylakoid membranes (II), and multiorganelle complexes (III), i.e., chloroplasts, mitochondria, peroxisomes, and cytoplasm surrounded by plasma membrane (from Larsson et al. 1985).

preparations by countercurrent distribution (Larsson et al. 1971; for a description of this automatic multistep procedure, see Albertsson 1986). The membrane fraction having the highest affinity for the polyethylene glycol-rich upper phase was identified as multiorganelle complexes, comprised of different cell organelles (chloroplasts, mitochondria, peroxisomes) and cytoplasm surrounded by plasma membrane (Fig. 1). This high affinity for the upper phase was later exploited for the preparation of plasma membrane from corn coleoptiles (Widell and Larsson 1981).

Purifying plasma membranes from a plant material not used before first necessitates the optimization of the phase system. By varying the polymer concentration and the salt composition of the phase system, optimal conditions for separation are obtained. This is conveniently done by first varying the polymer concentration in a series of phase systems where the salt composition is constant (Fig. 2A), and monitoring different membrane marker activities. According to the results so obtained, a suitable polymer concentration is chosen. Keeping the polymer concentration constant, a new series of phase systems is prepared, varying the salt composition (Fig. 2B). With this approach an optimal two-phase system can be tailored (Larsson et al. 1986).

The effects of polymer and salt concentration on the partition of different membranes may be explained as follows.

(i) Close to the critical polymer concentration almost all membrane particles are distributed to the upper phase. With increasing polymer concentration the membrane vesicles start to collect at the interface. This response is more pronounced for membrane vesicles of intracellular origin than for plasma membranes. The reason for this is probably related to differences in hydrophobic/hydrophilic properties of the different membranes (Albertsson 1986), since the polyethylene glycol concentration in the upper phase increases, which thus becomes more hydrophobic.

(ii) An interfacial electrical potential is created by adding ions (i.e., salt) to the phase system (Johansson 1970, Albertsson 1986). Popularized, HPO_4^{2-} , for example, has a stronger affinity for the Dextran-rich lower phase than K^+ or Na^+ have, thereby creating a "positive" upper phase. Negatively charged membrane vesicles are therefore increasingly "pulled" into the upper phase by raising the phosphate buffer concentration in the phase system. The same effect is obtained with other di- or trivalent anions like maleate, citrate and sulphate. Monovalent anions (e.g., Cl^- and acetate) have the opposite effect, creating a "negative" upper phase, and thereby "pushing" negatively charged membranes down to the interface.

These polymer and salt effects explain the distribution of the different membrane marker activities in the optimization procedure given above (Fig. 2A, B). The different effects on different membranes of increasing polymer and salt concentrations are probably due to differences in hydrophobic/hydrophilic properties as well as in surface net charge density between membranes.

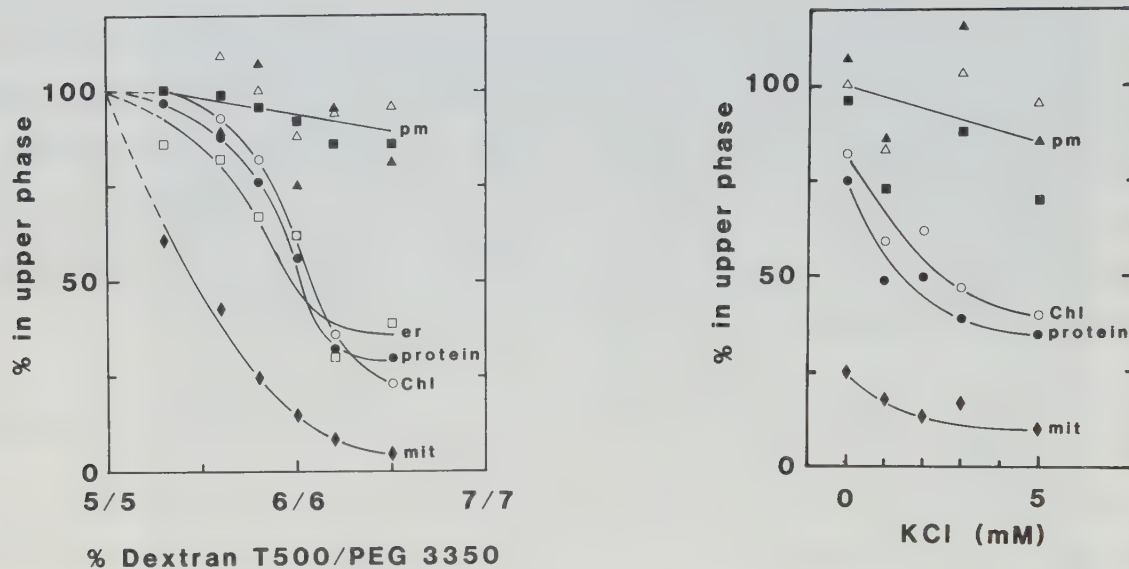


Fig. 2A, B. Effects of increasing polymer (A) and Cl^- -concentrations (B) on the partition of different membranes in a microsomal fraction from oat leaves. Markers used in A: Cytochrome c oxidase ($\blacklozenge$) for mitochondria, chlorophyll ($\circ$) for chloroplast thylakoids, antimycin A-insensitive NADPH-cytochrome c reductase ($\square$) for ER; and for the plasma membrane, total K^+ -stimulated, Mg^{2+} -dependent ATPase ($\blacksquare$), total vanadate-inhibited K^+ , Mg^{2+} ATPase ($\blacktriangle$), and latent K^+ , Mg^{2+} ATPase ($\triangle$). Apart from the polymers the phase systems contained 0.33 M sucrose and 5 mM K-phosphate, pH 7.8. Temperature 4°C . Markers used in B: As in A, except that latency of the vanadate inhibition of the K^+ , Mg^{2+} ATPase ($\triangle$) was used instead of latency of the whole activity. Apart from KCl, the phase system contained 5.8 % Dextran, 5.8 % polyethylene glycol, 0.33 M sucrose, and 5 mM K-phosphate, pH 7.8. Temperature 4°C .

A batch-procedure is usually employed when preparing plasma membranes from plant tissue. A microsomal membrane pellet (prepared as described above) is usually the starting material. This pellet is suspended in an appropriate isoosmotic medium and added to a tube containing a pre-weighed phase mixture, to give a phase system of optimal composition (Fig. 3). The phase system is well mixed (e.g., by 20 to 30 inversions of the tube) and phase settling is facilitated by low-speed centrifugation in a swing-out-bucket centrifuge for a few minutes (for a detailed treatise, see I and Larsson et al. 1986). Thereafter, approximately 90 % of the upper phase (U_1) is removed,

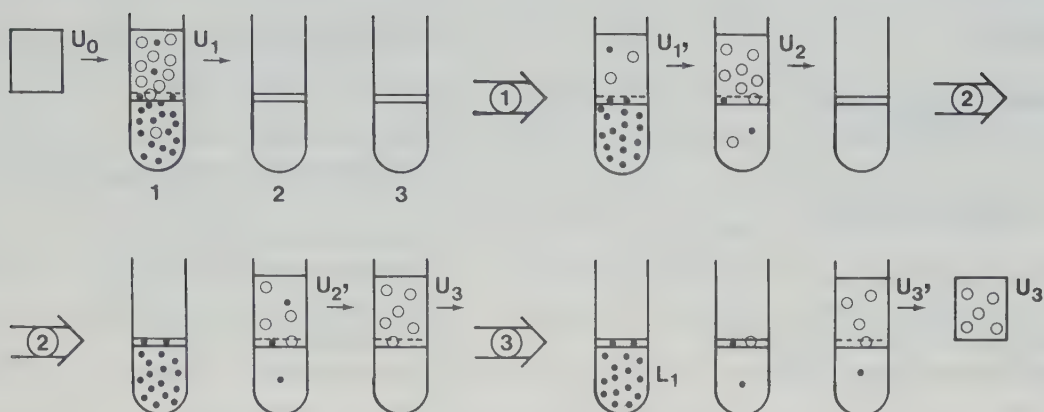


Fig. 3. Separation of plasma membranes (○) and intracellular membranes (●) by a batch-procedure of three steps.

taking care not to disturb the material at the interface, and re-partitioned twice with fresh lower phase, giving U_3 . In order to increase the yield of plasma membranes, the lower phase may be re-extracted, in sequence, with fresh upper phase (U_0), giving U_3' . The upper phases may, of course, be re-partitioned more than twice, if necessary. Fresh upper and lower phases are obtained from a bulk phase system of identical composition. The bulk phase system is prepared separately and equilibrated at the desired temperature before separating the phases. Note that there is a true equilibrium between the upper and the lower phase, and the volumes of fresh phases are therefore not critical and can be added by eye.

By using aqueous two-phase systems plant plasma membranes have been obtained from several plant species and organs (Tab. 2). From the investigations done so far, a general pattern in the partition behavior of plant membranes can be deduced. Thus, the affinity for the upper phase increases in the following order: inside-out thylakoid membrane vesicles < intact chloroplasts < mitochondria $\approx$ peroxisomes < endoplasmic reticulum < thylakoids $\approx$ Golgi membrane vesicles $\ll$ multiorganelle complexes = protoplasts = plasma membrane vesicles (Albertsson et al. 1982, Larsson 1983).

Tab. 2. Plasma membranes from different plant materials purified by aqueous polymer two-phase systems.

Plant material	References
Barley leaves, light-grown	I III Rochester et al. (1986a,b)
Spinach leaves, light-grown	I Rochester et al. (1986b)
Oat leaves, light-grown	Larsson et al. (1986)
Lemna gibba, light-grown	Ingmarsson et al. (1986)
Scenedesmus obliquus, light-grown	Palmkvist et al. (1986)
Scots pine needles, light-grown	Hellergren et al. (1983)
Barley roots	III
Oat roots	Widell et al. (1982) II Møller et al. (1984) Møller and Lundborg (1985a,b) Sommarin et al. (1985) Bérczi and Møller (1986) Lomax et al. (1986)
Wheat roots	Lundborg et al. (1981) Bérczi et al. (1984) Møller and Lundborg (1985a,b) Sommarin et al. (1985) Møller and Bérczi (1985,1986) Bérczi and Møller (1986)
Cucumber roots	Memon et al. (1987)
Maize roots	Buckhout and Hrubec (1986)
Maize shoots, etiolated	Clément et al. (1986a,b)
Rye, crown tissue	Uemura and Yoshida (1983)
Orchard grass, crown tissue	Yoshida et al. (1983) Yoshida and Uemura (1984)
Corn coleoptiles	Widell and Larsson (1981)
Mulberry bark	Yoshida (1984)
Soybean hypocotyls	Barr et al. (1985b) Sandelius et al. (1986)
Mung bean seedlings, etiolated	Yoshida et al. (1986)
Oat leaves, etiolated	Widell et al. (1982)
Cauliflower inflorescences	Widell and Larsson (1981) Widell et al. (1983) Caubergs et al. (1983) II IV Rochester et al. (1986b) Uemura and Yoshida (1986)
Jerusalem artichoke tubers	

3. Plasma membrane markers

3.1. The marker concept

A marker is any tag that can be used to identify a cell component and distinguish it from other cell components during and/or after a fractionation procedure. It can either be an endogenous property (e.g., an enzyme activity) or exogenously imposed (e.g., a surface label). The ideal marker is uniquely distributed to a single cell component, but this is rather the exception than the rule. Most membrane markers have one primary location and one, or several, secondary locations. In order to establish the purity of a plasma membrane preparation it is not only necessary to monitor several plasma membrane markers but also to monitor markers of cell components that might contaminate the preparation. Furthermore, it is an advantage to choose as disparate plasma membrane markers as possibly, that is, markers whose functions are based on totally different properties of the membrane.

Markers can roughly be classified as either morphological, cytochemical or biochemical.

3.2. Morphological markers

Some organelles and membranes have a morphology that can be used for identification. Microsomal fractions composed of membrane vesicles do not leave much room for the use of morphological markers. Exceptions are the different thickness of membranes, and the fact that the unit-membrane structure (i.e., the dark-light-dark pattern in stained sections) is more or less pronounced for different membranes. Using thin sections and the staining method of Franke et al. (1969), involving a simultaneous fixation with glutaraldehyde and osmium tetroxide enhancing the unit-membrane structure, it was found that the plasma membrane is thicker (~10 nm) than are the intracellular membranes (~5 to 9 nm) and that the dark-light-dark pattern is more distinct for the plasma membrane than for other membranes (Sandelius et al. 1986).

3.3. Cytochemical markers

Specific staining with phosphotungstic acid-chromic acid (PTAC) at low pH (ca 3) seems to be the most widely accepted marker for the plant plasma membrane (Roland et al. 1972, Roland 1978, Nagahashi et al. 1978, Uemura and Yoshida 1983, Yoshida et al. 1983, 1986, Yoshida 1984, Sandelius et al. 1986). This method probably involves the oxidation of plasma membrane associated glycolipids or glycoproteins (by periodic acid) and the subsequent reaction of the aldehydes formed with PTAC (for a detailed treatise, see Roland 1978). It has also been suggested (Roland 1978) that staining is more effective and resolution improved with silicotungstic acid (STA) rather than PTA. The usefulness of STA-staining has been shown in several recent papers (I, Widell et al. 1982, Widell and Larsson 1983, Hellergren et al. 1983, Buckhout and Hrubec 1986). The use of chromic acid in the PTAC-staining procedure is, to my knowledge, only directed to achieve a low pH. In our version of the STA-staining procedure a low pH is reached by using silicotungstic acid, and not the salt, and if the pH has to be adjusted, HCl or NaOH is used. Thus, the use of chromic acid, which might cause unspecific staining, is avoided.

Since the basis for the selective staining of plasma membranes with PTAC and STA probably is the heavy glycosylation of plasma membrane lipids or possibly proteins (Kjellbom and Larsson unpublished results, Yunghans et al. 1978, Yoshida 1984, Yoshida and Uemura 1984, Uemura and Yoshida 1986, Rochester et al. 1986, Yoshida et al. 1986), one may also expect ER and Golgi membrane vesicles to stain with these methods since the membrane flow concept (Morré and Mollenhauer 1974) and knowledge concerning sites of glycosylation (ER, proximal glycosylation; Golgi apparatus, terminal and O-linked glycosylation) point at this possibility. Thus, secretory membrane vesicles which are about to fuse with the plasma membrane react positively with PTAC (Vian and Roland 1972, Morré and Mollenhauer 1983).

The purity of plasma membrane fractions can thus be estimated from electron micrographs of material stained with a plasma membrane specific stain (PTAC or STA) by counting well stained and weakly stained vesicles.

3.4. Biochemical markers

3.4.1. K^+ -stimulated, Mg^{2+} -dependent ATPase

Several ATP-hydrolyzing activities are present in plant cells, such as acid phosphatases, alkaline phosphatases, apyrases (ATP diphosphohydrolases), an ATPase associated with the inner membrane of the chloroplast envelope (McCarty et al. 1984), a Golgi membrane ATPase (Chanson et al. 1984, Chanson and Taiz 1985, Ali and Akazawa 1986), a tonoplast ATPase (O'Neill et al. 1983), the F_1F_0 -type ATPases of mitochondria and chloroplasts, and a plasma membrane ATPase (Hodges and Leonard 1972, Sze 1985). The plasma membrane ATPase is K^+ -stimulated and Mg^{2+} -dependent and it is inhibited by orthovanadate but insensitive to oligomycin and azide (mitochondrial ATPase inhibitors; Serrano 1985, Sommarin et al. 1985, Sze 1985), and to molybdate (an inhibitor of acid phosphatases; Serrano 1985, Sommarin et al. 1985, Sze 1985). Like several ATPases of other origins (Serrano et al. 1986, Waldenhaus et al. 1985) the plant plasma membrane ATPase generates a phosphorylated intermediate. The stimulatory effect of K^+ is suggested to be due to increased dephosphorylation and thereby increased turnover of this phosphoenzyme (Briskin and Leonard 1982b, Briskin and Pool 1983a, b, Vara and Serrano 1983). Orthovanadate is thought to be inhibitory by competing with phosphate, thus preventing the formation of the phosphoenzyme intermediate (Briskin and Leonard 1982b). The substrate for the plasma membrane ATPase is most probably Mg-ATP. Thus, according to differences in pH optimum and ion-requirement, together with the influence of different known ATPase inhibitors, it is possible to distinguish the plasma membrane ATPase from other ATPase activities (Gallagher and Leonard 1982), especially from the Cl^- -stimulated, NO_3^- -sensitive ATPase of the tonoplast (i.e., the vacuolar membrane). Often only the K^+ -stimulated increment of the plasma membrane ATPase is used as a plasma membrane marker, since several different Mg^{2+} -ATPases are present in plant cells. Furthermore, the addition of detergent is essential for maximum activity (see section 5.1.; I, II, III, Larsson et al. 1986).

3.4.2. Glucan synthetase II

The presence of different polyglucans (e.g., callose and cellulose) at the surface of plant cells implicate the plasma membrane as a potential site of synthesis. Using micromolar concentrations of UDP-glucose as substrate, Ray et al. (1969) could demonstrate a Golgi-associated glucan synthetase activity. Using millimolar UDP-glucose concentrations, Van Der Woude et al. (1974) could demonstrate that another, but similar, glucosyl transferase activity was associated with the plasma membrane. The different glucan synthetase activities are since then termed GS I and GS II, respectively. As cellulose is a 1,4-linked β -D-glucan and callose is a 1,3-linked β -D-glucan and since the plasma membrane is implied to be the site of synthesis of both cellulose and callose, the plasma membrane is expected to carry both 1,4- and 1,3- β -glucan synthetase activities. In both the GS I and GS II assays a mixture of 1,4- and 1,3-linkages are produced according to enzymatic degradation and methylation analysis (Ray 1977, Henry et al. 1983). GS I is usually assayed with ca 1 μ M UDP-glucose and 10 mM Mg^{2+} present, while GS II is assayed without Mg^{2+} and with ca 0.1 mM UDP-glucose (Ray 1977, 1979). 1,3-Linkages are favoured by the addition of high (mM) concentrations of UDP-glucose, while the addition of Mg^{2+} increases the overall incorporation of glucose, but at the same time favours the formation of 1,4-linkages over 1,3-linkages (for a review, see Robinson 1985). According to a recent study using a partly purified membrane fraction from petiole tissue of sugar beet (Morrow and Lucas 1986), the incorporation of UDP-glucose is greatly favoured by the addition of divalent cations (5 mM $MgCl_2$ plus 5 mM $CaCl_2$) when using a high (0.1 mM) UDP-glucose concentration; the product being a linear 1,3- β -glucan. It was furthermore demonstrated; (i) that the 1,3- β -glucan synthetase required Ca^{2+} for maximal activity; (ii) that in the presence of 5 mM Mg^{2+} , a 10- to 15-fold activation was obtained by the addition of Ca^{2+} ; (iii) that in the absence of Mg^{2+} , a 30- to 40-fold activation by Ca^{2+} was observed. Similar results were obtained with a microsomal fraction from soybean cells (Kauss and Jeblick 1986).

The GS II activity requires the addition of a primer like cellobiose or sucrose (Ray 1979). However, a high concentration of primer may be inhibitory. Cellobiose at 0.3 M, as suggested by Ray (1979), seems to be too high (I, Kjellbom and Larsson unpublished observation). Cellobiose concentrations in the millimolar range is probably sufficient, as shown by Morrow and Lucas (1986). Furthermore, a suitable detergent stimulates the activity of GS II and 1,3- β -glucan

synthetase (II, Heiniger 1983, Larsson 1985, Kauss and Jeblick 1986, Larsson et al. 1986).

As has been pointed out (Morré & Buckhout 1979, Widell et al. 1982), the GS I activity is usually at least one order of magnitude lower than the GS II activity in the microsomal fraction, and is therefore not a useful marker for Golgi membranes in plasma membrane fractions due to the unavoidable overlap in the GS I and GS II assays. Despite the fact that GS II activity is likely also to be associated with intracellular membranes (Ray 1979), and despite the uncertainty concerning the identity of the product formed (i.e., 1,3- β - and/or 1,4- β -linkages) and uncertainty of optimal assay conditions, GS II activity is a useful marker for the plasma membrane and shows a severalfold enrichment in plasma membrane fractions (I, II, Larsson et al. 1986, Sandelius et al. 1986). GS II has also been firmly correlated with specific staining of plant plasma membranes (I, Van Der Woude et al. 1974, Widell et al. 1982, Yoshida et al. 1983).

3.4.3. Blue-light-reducible b-type cytochrome

The presence of a blue-light-reducible b-type cytochrome in higher plants was shown by Brain et al. (1977). This cytochrome was suggested (Senger and Briggs 1981) and then shown (I, IV, Leong and Briggs 1981, Leong et al. 1981, Lundborg et al. 1981, Widell and Larsson 1981, 1983, Widell et al. 1982, 1983) to be located in the plasma membrane. The light-induced absorbance change (LIAC) accompanying the reversible reduction of this b-type cytochrome can therefore be used as a marker for the plasma membrane, but as for most other plasma membrane markers, its location is not solely restricted to the plasma membrane. It can also be found in intracellular membranes (probably the ER; Widell and Larsson 1983, Widell et al. 1983). The α -band (558 nm) and β -band (529 nm) typical for this b-type cytochrome are shown in Fig. 4. LIAC is assayed as the difference between the maximum and minimum absorbance in the Soret-region of the light-reduced minus dark (oxidized) difference spectrum, i.e., $\Delta(A_{428}-A_{412})$ in curve D in Fig. 4. LIAC can either be measured in the presence of methylene blue or using the endogenous photo-receptor (presumably a flavin; for a detailed treatise, see Widell 1986).

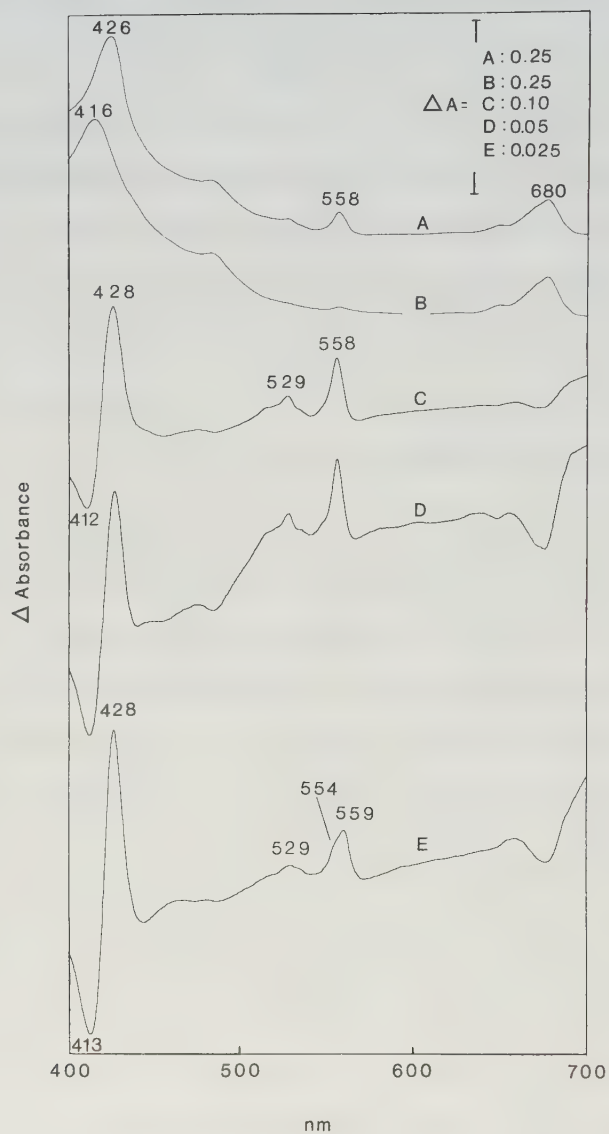


Fig. 4. Low-temperature spectra of plasma membrane fractions from leaves of spinach (A-D) and barley (E). A, dithionite-reduced; B, oxidized; C, dithionite-reduced minus oxidized; D, blue-light-reduced minus oxidized; E, dithionite-reduced minus oxidized.

3.4.4. UDP-glucose sterol β -glucosyl transferase

Glycosylation of lipids has been implied to be associated with plant plasma membranes (Van Der Woude et al. 1974, Hartmann et al. 1977, Hartmann-Bouillon and Benveniste 1978, Hartmann-Bouillon et al. 1982, Chadwick and Northcote 1980, Quantin et al. 1980, Henry et al. 1983) and may thus be used as a plasma membrane marker. However, this activity is also found in intracellular membranes (e.g., Sandelius et al. 1986). Whether the endogenous substrate is sterols is not clear. Recent observations indicate that sterol glycosides and acylated sterol glycosides are not present in all plant plasma membranes, whereas glucocerebroside has been found in all pure plant plasma membranes so far examined (see section 5.5., Tab. 3)(Rochester et al. 1986a, b). The glucocerebroside seems to be a major constituent of the plant plasma membrane, comprising 8-22 mol % of total lipid in plasma membranes of different species (Rochester et al. 1986a,b). Sterol is added in the UDP-glucose sterol β -glucosyl transferase assay, and the total incorporation of UDP- ^{14}C -glucose into the chloroform-methanol-soluble fraction is taken as a measure of the enzyme activity (Hartmann et al. 1977, Sauer and Robinson 1985). Whether it is sterols or ceramide that are glycosylated *in vivo* is impossible to state. Thus, glucocerebroside may well be a product of the UDP-glucose sterol β -glucosyl transferase, especially in plasma membranes where no sterol glycosides or acylated sterol glycosides are present.

3.5. Non-enzymatic markers for the plasma membrane

3.5.1. High sterol to phospholipid ratio

Plant plasma membranes, as well as animal plasma membranes, have a high sterol to phospholipid ratio compared to intracellular membranes (see section 5.5, Tab. 3)(Leonard and Hodges 1980, Hall 1983, Yoshida and Uemura 1984, Rochester et al. 1986a). This property may only be used as a complement to other plasma membrane markers. It should certainly not be used in comparing and estimating the purities of plasma membrane preparations from different plants and/or organs since the lipid composition of plasma membranes evidently differ considerably (see section 5.5., Tab. 3)(III, Wallach and Lin 1973).

3.5.2. High partition in aqueous polymer two-phase systems

That plasma membrane vesicles have a high partition in aqueous polymer two-phase systems was first shown by Larsson et al. (1971) when subfractionating a crude chloroplast pellet using countercurrent distribution. This unusual high affinity for the upper phase has since been verified for an increasing number of different plant plasma membranes. The notion that plasma membranes must possess some kind of surface property which sets them apart from intracellular membranes is strengthened by the observation that plasma membrane vesicles and protoplasts have the same high affinity for the polyethylene glycol-rich upper phase. This correlation has been shown for a large number of different plant materials of C₃, C₄ and CAM origin (Kanai and Edwards 1973a,b, Huber and Edwards 1975, Perlin and Spanswick 1980, Huber and Moreland 1980, 1981, Hallberg and Larsson 1981). Thus, a high partition in aqueous two-phase systems can be regarded as a true property of the plasma membrane (I) and be used as a marker (Widell and Larsson 1981).

3.6. Surface binding and labeling

A ligand binding property often used to identify the plant plasma membrane is the binding of the auxin transport inhibitor 1-N-naphtyl-phthalamic acid (NPA) (Lembi et al. 1971, Thomsen et al. 1973, Ray 1977, Hartmann-Bouillon and Benveniste 1978, Jacobs and Hertel 1978, Rubery 1981, Uemura and Yoshida 1983). However, the plasma membrane is evidently not the only site for NPA-binding, since ER fractions also display substantial binding (e.g., Sandelius et al. 1986). This , at least dual, distribution of NPA-binding is not surprising since auxin is known to be transported through the plasma membrane, and may thus have its primary site of action at an intracellular membrane. Anyhow, the validity of NPA-binding as a plasma membrane marker has been shown in several reports, and the binding correlates with other plasma membrane markers, e.g., PTAC-staining (Lembi et al. 1971, Nagahashi et al. 1978, Uemura and Yoshida 1983, Sandelius et al. 1986).

Binding of the fungal toxin fusaric acid, a growth-promoting toxin, is also utilized as a plasma membrane marker since it is thought to act preferentially at the plasma membrane (Dohrman et al.

1977, Stout and Cleland 1980). It has not been used extensively and therefore it is difficult to judge its usefulness as a plasma membrane marker.

The presence of Con A-binding sites on the outer surface of plant protoplasts and on plasma membrane vesicles has been demonstrated, thereby indicating the presence of mannosyl and/or glucosyl residues on the outer surface of the plasma membrane (Burgess and Lindstead 1976, 1977, Williamson et al. 1976, Larkin 1978, Boss and Ruesink 1979, Chin and Scott 1979, Williamson 1979, Travis and Berkowitz 1980, Berkowitz and Travis 1982, Hartmann-Bouillon et al. 1982, Hartmann et al. 1983a,b). The cisternal face of vesicles of the ER will also bind Con A (Berkowitz and Travis 1981, Hartmann-Bouillon et al. 1982, Hartmann et al. 1983a,b). It is also to be expected that lectin binding sites will be detected in vesicles of the Golgi apparatus, since this is a well-known site of glycosylation (Morré 1975).

Plasma membrane proteins exposed at the outer surface of a protoplast comprise a small portion (probably less than 0.1 %) of total cell protein, rendering labeling and binding studies heavily dependent on the intactness of the protoplasts. As in the case of using intact protoplasts for studies on plasma membrane associated events, only a few broken or leaky protoplasts will jeopardize selective binding or labeling.

Several other labeling techniques can be used to follow the plasma membrane when preparing plasma membranes from protoplasts. The same restrictions are applicable in these cases as those mentioned for binding of NPA, fusaric acid and Con A, but now even more pronounced since these other techniques do not have the same specificity.

Lactoperoxidase-catalyzed iodination of tyrosine groups (Schribeci et al. 1982), as well as modification of tyrosine, histidine or primary amino groups with labeled diazotized sulphanilic acid (Galbraith and Northcote 1977, Chadwick and Northcote 1980, Perlin and Spanswick 1980), are such techniques for labeling of plasma membrane proteins at the outer surface of protoplasts. Labelled lectins may also be used for binding to intact protoplasts prior to homogenization and purification of plasma membranes (Boss and Ruesink 1979). Furthermore, the GS II activity has been used for labeling plasma membranes. Incubation with UDP-¹⁴C-glucose produces labeled callose, which remains attached to the plasma membrane during subsequent homogenization and fractionation (Andersson and Ray 1978, Raymond et al. 1978). When incubating protoplasts with

lanthanum salts, La^{3+} is reported to bind irreversibly to the outer surface of the plasma membrane. No redistribution of electron-dense La^{3+} is reported upon homogenization and the following fractionation procedure, and marker enzyme activities can still be assayed in the plasma membrane fraction despite the presence of La^{3+} (Taylor and Hall 1979, Hall and Taylor 1979).

4. Markers for contaminating membranes

To be able to estimate the contamination of intracellular membranes in plasma membrane fractions, it is of importance to have convenient and reliable markers for these membranes. However, the markers for most intracellular membranes tend to have more than one location. The only appropriate single location markers seem to be cytochrome c oxidase of the inner mitochondrial membrane and chlorophyll of the thylakoid membrane of chloroplasts.

4.1. The tonoplast

In fully differentiated higher plant cells the vacuole is by far the largest organelle, occupying as much as 90 % of the total cell volume (Robinson 1985). The vacuolar membrane, i.e., the tonoplast, can be distinguished from other cell membranes due to the presence of a Cl^- -stimulated, NO_3^- -sensitive Mg^{2+} -ATPase. It is neither stimulated by monovalent cations (e.g., K^+), nor is it inhibited by orthovanadate like the plasma membrane associated ATPase (Walker and Leigh 1981, O'Neill et al. 1983). As the native substrate is Mg-ATP, it is dependent on Mg^{2+} like the plasma membrane ATPase. The tonoplast ATPase is insensitive to azide and oligomycin and can thus be distinguished from the mitochondrial ATPase. Its insensitivity to molybdate sets it apart from acid phosphatases. The tonoplast ATPase, as the plasma membrane ATPase, is an electrogenic proton pump, translocating protons across the tonoplast into the vacuole (for a review on proton-pumping ATPases, see Sze 1985).

4.2. The endoplasmic reticulum

Antimycin A-insensitive NADPH-cytochrome c reductase is a well-established marker for the ER of plant cells (Lord et al. 1973, Quail 1979). However, recent reports show that this activity also is present in plant plasma membrane preparations as a true property of the plasma membrane and not due to contamination by ER (I, IV, Lundborg et al. 1981, Larsson 1985). The significance of this redox activity of the plant plasma membrane will be discussed later in relation to blue-light

photomorphogenesis (I, IV). Furthermore, indications of NADPH-cytochrome c reductase activity in Golgi membranes of animal cells have been presented (Howell et al. 1978, Jarasch et al. 1979).

4.3. Golgi membranes

Latent IDPase activity is often used as a marker for membranes of the Golgi apparatus (Ray et al. 1969, Hardin et al. 1972, Morré et al. 1977, Morré and Buckhout 1979, Ray 1979, Green 1983). Latent IDPase is either assayed in plant membrane fractions which have been stored at 0 to 4°C for a few days, or by inclusion of detergents (e.g., Triton X-100) in the assay medium. From the activity thus obtained the non-latent IDPase activity of fresh samples is subtracted. It is the increment caused by storage or by addition of an amphiphilic agent that is taken as the latent activity. Non-latent IDPase activity is often present in other membrane fractions than those of Golgi membranes, e.g., in plasma membrane fractions (III, Morré and Buckhout 1979, M'Voula-Tsieri et al. 1981), and can either be due to a truly plasma membrane associated non-latent IDPase or to a lack of substrate specificity for the plasma membrane ATPase (Yoshida et al. 1983), although most of the latter activity should be latent (see sections 5.1. and 6.1.1.). Latent IDPase activity in ER fractions has also been reported (M'Voula-Tsieri et al. 1981). In some cases no latency of the IDPase activity of Golgi or microsomal fractions was detected (III, Nagahashi and Beevers 1978, Koehler et al. 1976), possibly due to low Golgi content in the plant material used (III).

One additional enzyme marker often used for the Golgi apparatus is glucan synthetase I (GS I), usually assayed at low (micromolar) UDP-glucose and high (10 mM) Mg^{2+} concentrations (Ray 1979). As discussed previously, GS I activity is thought to form primarily 1,4- β -linkages, although most reports suggest that both 1,3- β - and 1,4- β -linkages are formed with the assay conditions used (Ray 1977, Henry et al. 1983). Several reports show GS I activity to be associated with morphologically identifiable dictyosomes (Ray et al. 1969, 1976, Brett and Northcote 1975, Bowles and Kauss 1976). However, other reports indicate a dual distribution of GS I activity, appearing in both Golgi and plasma membrane enriched fractions (e.g., Widell et al. 1982, Sturm and Kindl 1983). In view of the findings discussed above, and also because of the unavoidable overlap in the GS I and GS II assays, the conclusion is that GS I should not be used as a single positive marker for the Golgi apparatus (Morré and Buckhout 1979, Quail 1979, Ray 1979, Widell

et al. 1982).

The thickness of the membranes in the Golgi cisternal stack is intermediate of those of the ER and those of the plasma membrane, the latter being the thickest. This feature, together with a more pronounced unit membrane structure (i.e. dark-light-dark pattern) for plasma membranes, have been used to distinguish the Golgi membranes from plasma membranes and membranes of the ER (Sandelius et al. 1986).

Fucosyl transferase activity has been correlated with the presence of vesicles of the Golgi apparatus. Incorporation of labelled fucose (both in vivo and in vitro) does not appear to be restricted to Golgi membranes (Sturm and Kindl 1983). Fucosyl transferase activity and incorporation of labeled fucose can also be seen in presumptive plasma membrane and ER fractions. Despite this fact, fucosyl transferase seems to be as useful as GS I and latent IDPase as a marker for Golgi membranes.

4.4. Other possible contaminants

Contamination of plasma membrane fractions with adsorbed constituents of the vacuolar content and the cytoplasm may be monitored by the presence of α -mannosidase and malic enzyme activities, respectively (Martinoia 1981, Pupillo and Del Grosso 1981, Boller 1982). The chloroplast stromal protein ribulose biphosphate carboxylase/oxygenase, which may constitute over 50 % of soluble leaf protein, has recently been identified as a contaminant of leaf plasma membranes (Kjellbom and Larsson, unpublished results). It is probably adsorbed on the surface of all membranes upon homogenization of leaf tissue. The nuclear envelope may be distinguished from the plasma membrane due to RNA and DNA still remaining attached despite extensive processing. The contamination by microbodies may be monitored by the presence of catalase. The use of isocitrate lyase and NADH-hydroxypyruvate reductase as markers for the matrix of glyoxysomes and peroxisomes, respectively, might be more dependent of the choice of tissue and of developmental stage (Quail 1979, Robinson 1985).

5. Properties of isolated plasma membrane vesicles

Depending upon the technique used for the isolation of plant plasma membranes, the vesicles obtained may differ in sidedness, membrane permeability, isoelectric point, and surface net charge density.

5.1. Sidedness

Conflicting results regarding the sidedness of plasma membrane vesicles purified by sucrose density gradients have been obtained using Con A-binding (Hartmann et al. 1983a,b, Randall and Ruesink 1983), latency of nucleoside diphosphatase activity (M'Voula-Tsieri et al. 1981), fusicoccin binding (Hertel et al. 1983), and binding of 1-N-naphthyl phthalamic acid (NPA) (Michalke 1982). If plasma membrane vesicles of different orientation are produced during homogenization, vesicles of both orientations would be expected to band together, since density gradients purify membrane vesicles according to size and density, and should therefore not distinguish between vesicles of different sidedness.

Assuming that the Mg-ATP binding site of the plasma membrane associated K^+ -stimulated, Mg^{2+} -dependant ATPase is located on the cytoplasmic side of the membrane, the increase in enzyme activity upon addition of Triton X-100 shows that plasma membrane vesicles purified by aqueous polymer two-phase systems are right side-out (Fig. 5) (II, III, Larsson et al. 1986). This orientation is supported by the fact that multiorganelle complexes (Larsson et al. 1971, Larsson and Andersson 1979) and protoplasts (e.g., Hallberg and Larsson 1981) also have a high affinity for the upper phase. Electron spin resonance (ESR) measurements, on oat plasma membrane vesicles obtained by two-phase partition also support a right side-out orientation (Lomax et al. 1986).

As free-flow electrophoresis separates membrane vesicles according to differences in surface net charge density, one of two criteria on which two-phase separation is based, plasma membrane vesicles obtained by free-flow electrophoresis may also be right side-out. Experiments aimed at assigning the sidedness to plant plasma membrane vesicles purified in this way have not yet been reported. A right side-out orientation is strengthened by the fact that the different sides of the plant plasma membrane seem to differ considerably in surface net charge density (III, Møller et al.

1984). Vesicles of different orientation should therefore exhibit quite different behaviour in free-flow electrophoresis as well as in two-phase systems.

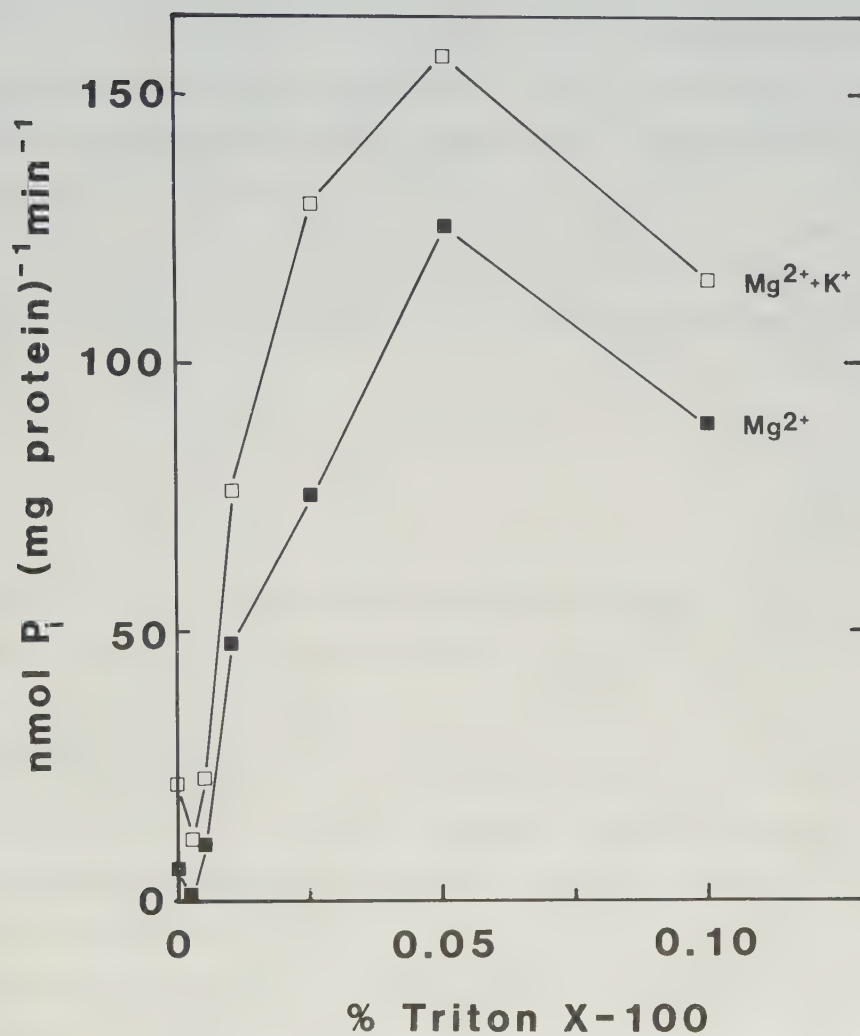


Fig. 5. Latency of the plasma membrane ATPase activity on addition of Triton X-100. Mg^{2+} , 1 mM MgSO_4 ; $\text{Mg}^{2+} + \text{K}^{+}$, 1 mM MgSO_4 + 25 mM KCl.

5.2. Plasma membrane permeability

Assuming that the Mg-ATP binding site of the plasma membrane ATPase is located at the cytoplasmic side of the membrane, the degree of intactness of plasma membrane vesicles can be estimated (II, III, Bérczi and Møller 1986, Yoshida et al. 1986). The latency observed at 0.05 % Triton X-100 (Fig. 5) suggests that plasma membrane vesicles prepared from cauliflower inflorescences, using two-phase partitioning, are 90 % sealed and right side-out (II). Similar results were obtained by ESR measurements on oat root plasma membranes (Lomax et al. 1986). If the vesicles obtained by sucrose density gradient centrifugation are right side-out, the latency of K^+ , Mg^{2+} -ATPase (Randall and Ruesink 1983, Hodges and Mills 1986) indicates that about 50 % of these vesicles are leaky to Mg-ATP. Another interpretation of these results would be that sucrose density gradients produce about equal amounts of plasma membrane vesicles of opposite sidedness. The considerable and changing osmotic effect exerted on the vesicles in a sucrose density gradient may, however, well render the membranes leaky (II, Bérczi and Møller 1986, Hodges and Mills 1986).

5.3. Isoelectric point

Cross-partition is a very useful method for determining the isoelectric point of membrane vesicles (Albertsson 1986). Membrane vesicles are partitioned in two series of phase systems in which the pH is varied. An electrostatic potential difference between the phases is created by including a cationic polyethylene glycol derivative in one of the series (e.g., trimethylamino-polyethylene glycol, creating a "positive" upper phase) and an anionic polyethylene glycol derivative in the other series (e.g., polyethylene glycol-sulphonate, creating a "negative" upper phase). At the isoelectric point, the partition of the vesicles is independent of the electrostatic potential difference created by the charged polymers. This point is identical with the cross-point of the two curves (Fig. 6). Right side-out plasma membrane vesicles of barley leaves and roots both had an isoelectric point at about pH 3.6. This is in close agreement with the value of pH 3.4 obtained with the same method for spinach multiorganelle complexes exposing the outer surface of the plasma membrane to the medium (Westrin et al. 1983). However, these values are significantly lower than the pH 4.4 observed for plasma membranes of etiolated pea stem using isoelectric focusing (Griffing and Quatrano 1984). Using the method of cross-partition, relatively high isoelectric pH's were obtained

for different thylakoid membrane vesicles (pH 4.0 to 4.7) and intact chloroplasts (pH 4.5) of spinach (Åkerlund et al. 1979, Westrin et al. 1983), indicating that the outer surface of the plasma membrane has a lower isoelectric point than intracellular membrane surfaces. Furthermore, note the difference between root and leaf plasma membrane vesicles in the distribution between the upper and lower phases at the isoelectric point (cross-point; Fig. 6; discussed in section 5.5.).

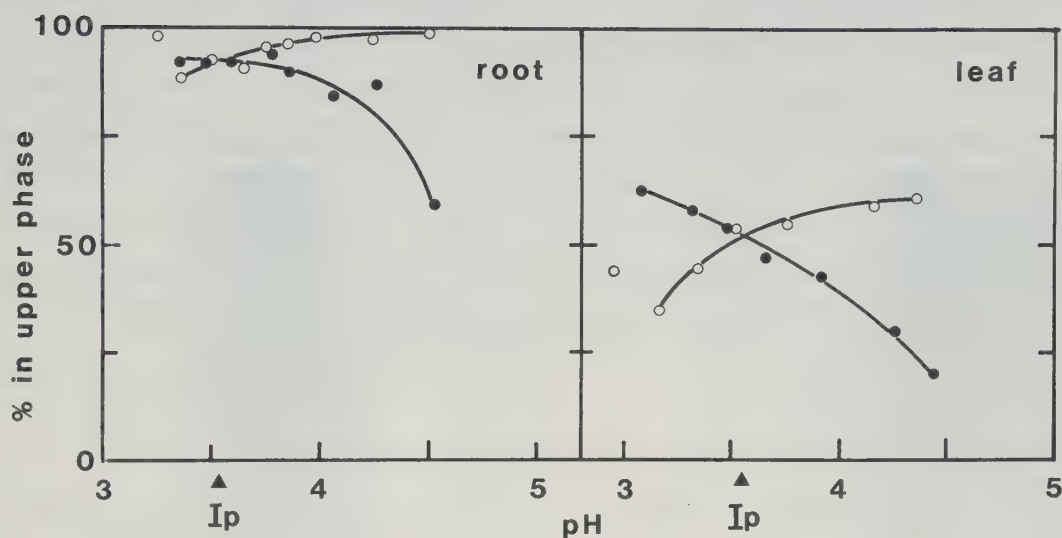


Fig. 6. Cross-partition of plasma membrane vesicles from barley roots and leaves.

5.4. Surface net charge density

The isoelectric point only gives information on the surface net charge density at that particular pH, since the surface net charge density is by definition zero at the isoelectric point. The surface net charge density of a membrane vesicle at other pH values can not be deduced from the isoelectric point. A useful technique for estimating the surface net charge density of membrane vesicles at other pH values is to measure the decrease in 9-aminoacridine fluorescence on addition of monovalent or divalent cations (see Bérczi et al. 1984 for a description of the method). According

to such measurements (at pH 7.0), the outer surface of plasma membrane vesicles from barley leaves was 50 % more negative (-29 mC m^{-2}) than that of root plasma membrane vesicles (-19 mC m^{-2}) (III). Furthermore, a clear tendency is that the estimated surface net charge density of the cytoplasmic side of plasma membrane vesicles is considerably more negative than the outer side (III; Møller et al. 1984). This suggests that right side-out and inside-out plasma membrane vesicles may be separated using methods based on differences in surface net charge density, such as free-flow electrophoresis and phase partition.

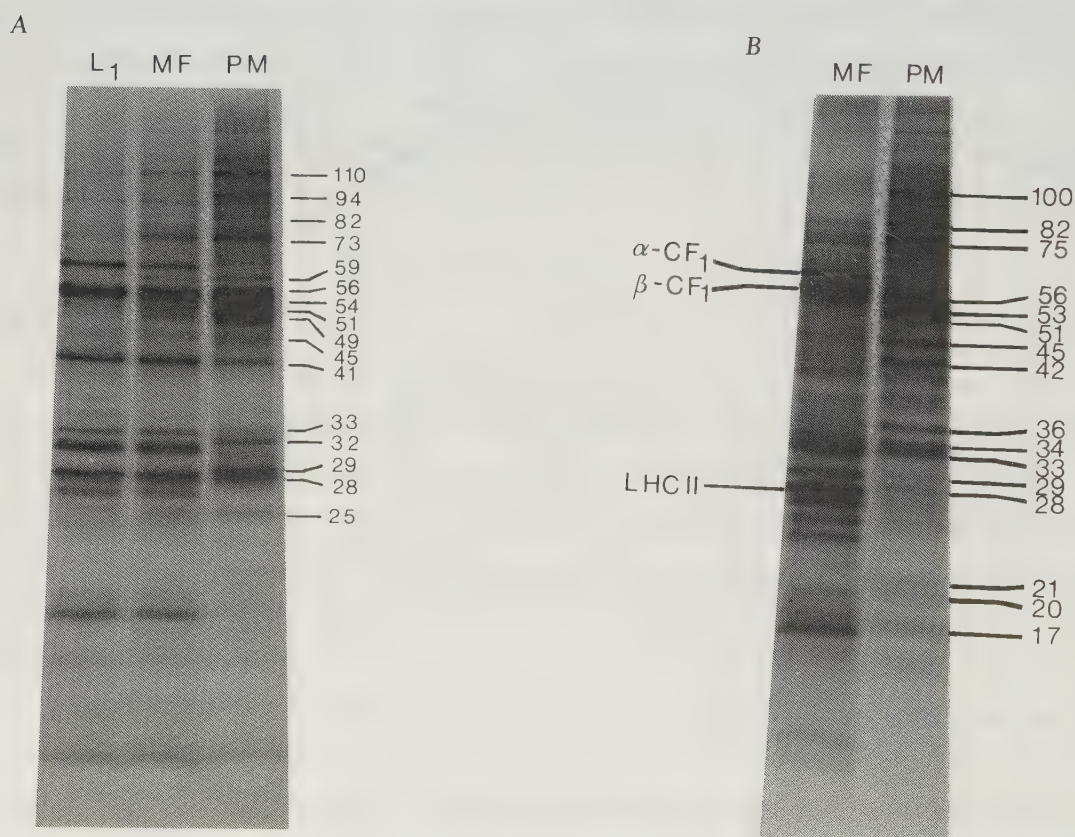


Fig. 7A, B. SDS-PAGE of membrane fractions of spinach (A) and barley (B) leaves. PM, plasma membrane fractions; MF, microsomal fractions; L₁, intracellular membrane fraction depleted in plasma membranes.

5.5. Protein and lipid composition

The plasma membranes of light-grown barley and spinach leaves contain about 30 and 40 % (w/w) protein, respectively (I). As a comparison spinach thylakoid membranes contain around 60 % (w/w) protein (Quinn and Williams 1983) and spinach leaf mitochondrial membranes contain around 77 % (w/w) protein (Gardeström et al. 1983). Thus, the plant plasma membrane contains relatively little protein compared to intracellular membranes. Some of the major polypeptides in the microsomal fraction from barley leaves can be identified as thylakoid polypeptides (Fig. 7B), and the complete absence of these polypeptides in the plasma membrane fraction underlines the efficiency of aqueous polymer two-phase systems in separating thylakoid membranes from plasma membranes. When comparing the polypeptide patterns of plasma membranes from roots and leaves of the same plant (III), a striking similarity is seen (Fig. 8). The only clear difference is the relative abundance of some of the polypeptides (arrowheads). This is an observation with important implications for differentiation and will be discussed below. When comparing the polypeptide composition of spinach (Fig. 7A) and barley (Fig. 7B) plasma membranes, it is evident that several of the prominent polypeptides of both species have approximately the same molecular weights.

One of the few plasma membrane polypeptides currently identified, the subunit of the K^+ -stimulated, Mg^{2+} -dependent ATPase which undergoes reversible phosphorylation, has an estimated molecular weight of ca 100 kDa in both oat roots (Vara and Serrano 1982, 1983), corn roots (Briskin and Leonard 1982a,b), and in red beet storage tissue (Briskin and Poole 1983c,d). This polypeptide has not yet been identified in spinach and barley.

Plasma membranes from light-grown spinach leaves have been purified by partition in an aqueous polymer two-phase system (I) and recently also by free-flow electrophoresis (Auderset et al. 1986). Upon SDS-PAGE large similarities in the polypeptide patterns were observed. Furthermore, apparently identical polypeptide patterns for plasma membrane fractions of soybean hypocotyls obtained by the two methods were recently reported (Sandelius et al. 1986). This suggests that plasma membrane fractions obtained by free-flow electrophoresis and two-phase partition are identical, or at least very similar.

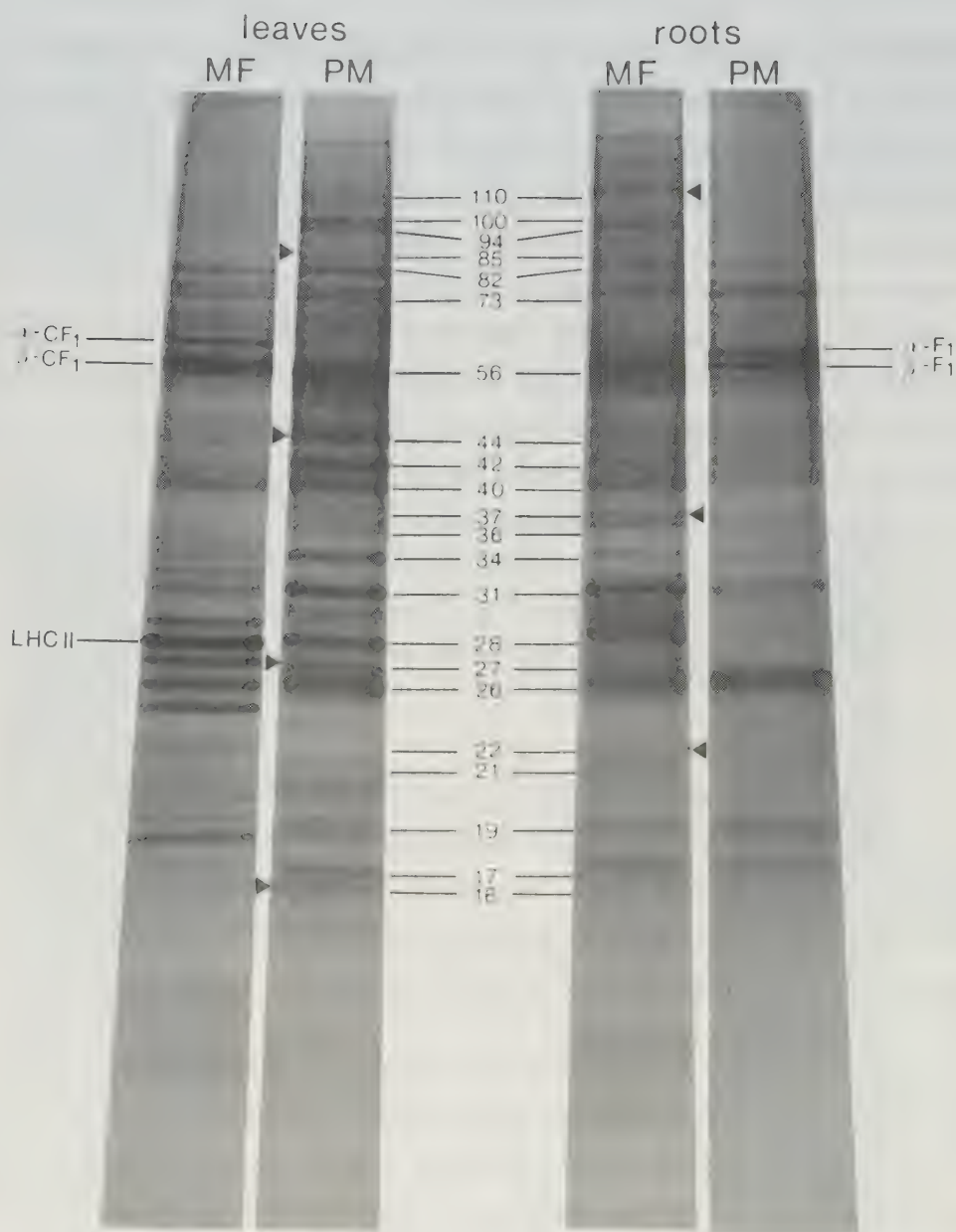


Fig. 8. SDS-PAGE of plasma membranes (PM) and microsomal fractions (MF) from barley leaves and roots. Differences between leaf and root plasma membrane polypeptide patterns are indicated by arrowheads.

In accordance with animal plasma membranes, the lipid bilayer of plant plasma membranes has been implied to have a high sterol to phospholipid ratio in comparison with intracellular membranes. Recent lipid analyses of highly purified plasma membrane preparations from barley leaves (Rochester et al. 1986a) and spinach leaves, cauliflower inflorescences and barley roots and leaves (Rochester et al. 1986b) support, and at the same time moderate, these findings. The sterol to phospholipid ratio is higher in the plasma membrane fraction than in other membrane fractions from the same tissue (Rochester et al. 1986a), whereas the ratio varies considerable between different species and organs (Tab. 3). These lipid analyses also include the novel finding of glucocerebroside, a glycosphingolipid, as a major constituent of the plant plasma membrane (Tab.3).

Tab. 3. Lipid composition of plasma membranes from spinach leaves, barley leaves, and barley roots.

	Lipid composition (mol %)		
	Spinach Leaf	Barley Leaf	Barley Root
Free sterols	7.0	34.6	57.2
Sterol esters	ND	ND	ND
Acylated sterol glycosides	12.9	ND	ND
Sterol glycosides	ND	ND	6.9
Monoglycosyldiacylglycerol	ND	3.7	1.0
Diglycosyldiacylglycerol	2.7	1.4	0.3
Sulphoquinovocyldiacylglycerol	ND	ND	ND
Glucocerebroside	13.6	16.3	8.6
Phosphatidylcholine	25.4	17.5	8.2
Phosphatidylethanolamine	24.4	10.8	6.8
Phosphatidic acid	6.1	2.1	0.8
Phosphatidylglycerol	3.1	9.2	1.7
Others ^a	4.9	4.0	8.6
<u>Free sterol</u>	0.1	0.8	2.2
Phospholipid			

a, Phospholipids migrating below phosphatidylcholine which were not individually assayed. May include phosphatidylinositol, phosphatidylserine and lysophospholipids. ND, not detected.
(Adapted from Rochester et al. 1986b)

In leaf plasma membrane fractions glucocerebroside constitutes as much as 14 to 22 mol %, depending on species and growth conditions. A notable observation is that even if the sterol composition of plasma membrane fractions from different species and organs varies considerably, the sterol composition of different membrane fractions from the same organ is relatively constant (Rochester et al. 1986a). Another notable observation is that sterol glycosides are found in plasma membranes of barley roots and cauliflower buds (Rochester et al. 1986b), but not in leaves of barley and spinach, albeit spinach contains large amounts of acylated sterol glycosides (Tab. 3).

Based on these findings it may be argued that a high sterol to phospholipid ratio can be used as a plasma membrane marker when comparing plasma membrane fractions with other membrane fractions within the same organ, but the ratio can evidently not be used for comparing the purity of plasma membrane fractions from different species or organs.

It is well known from studies on animal plasma membranes, especially the plasma membrane of erythrocytes, that different types of lipids and proteins are heterogeneously distributed transversely and laterally in the membrane (Hakomori 1986). This is most probably also the case with plant plasma membranes, as indicated by studies on for instance lectin binding (e.g., Hartmann et al. 1983a,b, Etzler 1985). Thus, the glycolipids are most probably all located in the outer leaflet of the membrane, as is the situation in animal plasma membranes (Hakomori 1986).

5.6. Comparison of preparations obtained with different methods

When comparing sucrose density gradient centrifugation, free-flow electrophoresis and two-phase partition as means of purifying plant plasma membranes it is quite evident that the two latter methods yield plasma membrane fractions of much higher purity (I, Bérczi and Møller 1986, Clément et al. 1986a, Hodges and Mills 1986, Sandelius et al. 1986). Based on STA-staining and marker enzyme analyses we have estimated the purity of plasma membranes prepared by phase partition to 90-100 % (Larsson et al. 1986). Assuming 95 % purity for the preparations produced by phase partition, a purity of ca 50 % is obtained both for oat root (Hodges and Mills 1986) and maize shoot (Clément et al. 1986a) plasma membranes prepared by sucrose density gradient centrifugation (calculated from the specific ATPase activities). It is also evident that plasma membrane vesicles purified by two-phase partition are sealed and right side-out (II, III), whereas

those prepared by sucrose density gradients are leaky (Bérczi and Møller 1986, Hodges and Mills 1986). Whether they are right side-out or of both sidedness is still at argument (M'Voula-Tsieri et al. 1981, Michalke 1982, Hartmann et al. 1983a,b, Hertel et al. 1983, Randall and Ruesink 1983). Besides being homogeneous with respect to sidedness, plasma membrane vesicles purified by two-phase partition, are homogeneous with respect to density, yielding only one band in continuous density gradients (Cauberg et al. 1983, Widell et al. 1983, Yoshida et al. 1983). Whether the plasma membrane vesicles produced by free-flow electrophoresis are right side-out and sealed has not yet been investigated. It is likely however, that they are sealed. This assumption is based on the fact that leakiness probably is due to high and changing osmotic pressure (i.e., low osmotic potential) exerted by sucrose in sucrose gradients. Thus, leakiness is not to be expected when using free-flow electrophoresis. This technique and two-phase partition both separate membrane vesicles according to differences in surface properties, albeit surface charge density plus hydrophobic/hydrophilic properties in the case of phase partition. The apoplastic surface of plasma membrane vesicles seems to have a less negative surface net charge density than the symplastic surface (III, Møller et al. 1984). This also points to a right side-out orientation of the plasma membrane vesicles corresponding to the least electronegative protein zone obtained by free-flow electrophoresis (Sandelius et al. 1986).

As can be judged from the few reports on purification of plant plasma membranes using free-flow electrophoresis, the purity of these plasma membrane fractions as compared to plasma membrane fractions purified by phase partition, can roughly be considered the same, at least 90 % pure (I,II,III, Buckhout and Hrubec 1986, Sandelius et al. 1986, Auderset et al. 1986). In a direct comparison of the two preparation methods, using soybean hypocotyls, identical polypeptide patterns were obtained for the plasma membrane preparations indicating a similar purity (Sandelius et al. 1986). When using green material, however, phase partition seems to be more effective, as shown by the complete removal of chlorophyll (i.e., thylakoid membranes) from the plasma membrane fraction of spinach leaves prepared by two-phase partition, whereas ca 21 µg chlorophyll per mg protein was still present after preparation by free-flow electrophoresis (Auderset et al. 1986). The recovery of plasma membranes, calculated as mg protein per 100 g homogenized fresh tissue, seems to be approximately the same for the two procedures (ca 1-10 mg, depending on species and growth conditions).

6. Biological functions

Our understanding of the structure and function of biological membranes has been improved significantly during the last two decades, with the proposal of the fluid mosaic membrane model (Singer and Nicholson 1972) as a stimulating and important impetus for further research. The current view of plasma membranes, chiefly derived from studies on membranes of animal origin, is that of a membrane composed mainly of polar lipids, constituting a more or less fluid bilayer, with associated proteins of different types. Hydrophilic proteins (i.e., peripheral) are thought to be located on the cytoplasmic side of the membrane and kept in place by hydrophilic interactions. Integral plasma membrane proteins have both hydrophilic and hydrophobic parts. The hydrophobic domains span the lipid bilayer while the hydrophilic ones protrude at each side of the membrane. The parts of the proteins projecting at the apoplastic side (i.e., the ectodomains) are thought to carry all oligosaccharides bound to plasma membrane proteins. Likewise, glycolipids are thought to be located in the outer leaflet of the plasma membrane. Cells have also developed means of controlling the lateral distribution of plasma membrane constituents. Some membrane surface receptors, for instance, are non-randomly distributed even before the binding of ligands (Nicolson 1982).

Recent experiments using Triton X-114-fractionation (Bordier 1981) and salt washing of spinach and barley leaf plasma membrane vesicles (Kjellbom et al. 1986b) indicate that the majority of the proteins are integral and that peripheral proteins are almost exclusively located on the cytoplasmic side of the plasma membrane. Furthermore, the lipid composition of these plasma membranes is similar to animal plasma membranes, e.g., being rich in sterols and phosphatidylcholine (see section 5.5.). Thus, plant plasma membranes appear to be similar in composition and structure to animal plasma membranes.

Some of the findings regarding plant plasma membrane activities imply certain biological functions of the membrane. Likewise, expected biological functions of the plant plasma membrane suggest that certain activities should be present in the membrane.

6.1. Transport-associated activities

6.1.1. Proton-translocating ATPases

The K^+ -stimulated, Mg^{2+} -dependent ATPase of the plant plasma membrane causes the extrusion of protons from the cell, whereas the Cl^- -stimulated, NO_3^- -sensitive ATPase of the tonoplast pumps protons into the vacuolar compartment (Fig. 9).

These ATP-driven proton pumping activities create electrochemical gradients across the membranes (Mitchell 1976, Nicholls 1982, for a recent review, see Sze 1985). The proton gradients generated are implicated in the active transport of several compounds such as sucrose (Briskin et al. 1984, Thom and Komer 1984, Wyse 1985), glucose (Suzuki 1982), organic acids (Marin et al. 1981, Kenyon and Black 1984, Lüttge et al. 1984), ions (Blumwald and Poole 1985, Schumaker and Sze 1984, Katz et al. 1986), and auxin (Hertel et al. 1983). Proton-translocating ATPases are important also in other physiological processes such as the regulation of cytoplasmic pH, stomatal movements, and thus gas exchange and water economy (Assman et al. 1985, Shimazaki et al. 1986), auxin-induced cell elongation (Cleland 1980, Gabathuler and Cleland 1985), and light-mediated responses (Racusen and Galston 1983).

Although, ATPases associated both with plasma membranes and tonoplasts are electrogenic proton pumps, they do not belong to the same family of ATPases. The plasma membrane H^+ -translocating ATPase shows a strong amino acid sequence homology with the K^+ -ATPase of *E. coli*, the (Na^+, K^+) -ATPase of kidney and the Ca^{2+} -ATPase of muscle (Walderhaug et al. 1985, Serrano et al. 1986). They all belong to a group of cation pumps known to form phosphorylated intermediates of the catalytic subunit (M_r ca 100 kDa). Thus, they appear to have evolved from a common ancestral ATPase. The stimulation of the plasma membrane ATPase by K^+ was previously taken as evidence for involvement of the enzyme in K^+ -transport. A more probable explanation is that K^+ stimulates dephosphorylation of the phosphorylated intermediate and thus an increased turnover rate of the enzyme (Briskin and Leonard 1982b).

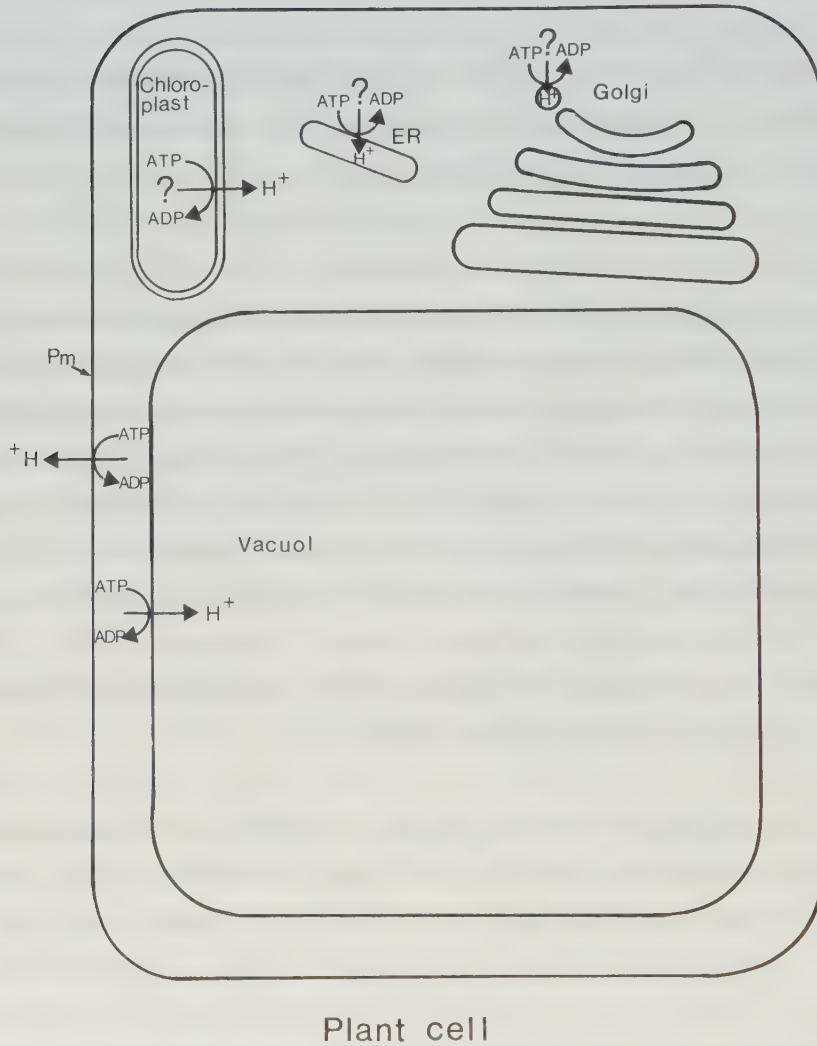


Fig. 9. Shown and proposed locations of different proton-pumping ATPases of plant cells (adapted from Sze 1985).

The tonoplast H^+ -translocating ATPase does not form a phosphorylated intermediate and has characteristics similar to those of F_1F_0 -type ATPases of chloroplasts, mitochondria and bacteria, although it is not quite homologous with these ATPases (Sze 1984, 1985).

6.1.2. Red-ox activities

NADPH and NADH oxidoreductases have been identified as constituents of most plant membranes (Møller and Lin 1986). An example is the often used enzymatic ER marker NAD(P)H-cytochrome c reductase, associated with the cytochrome P-450 system of ER, and involved in desaturation and hydroxylation reactions in mammalian cells. A number of reports on NAD(P)H redox activities located in plant plasma membranes have also appeared (I, Lin 1982, 1984, Deluca et al. 1984, Misra et al. 1984, Rubenstein et al. 1984, Barr et al. 1985a,b, Møller and Bérczi 1985, 1986, Thom and Maretski 1985, Buckhout and Hrubec 1986, Pupillo et al. 1986, Rubenstein and Stern 1986; for reviews, see Crane et al. 1985a,b, Lüttge and Clarkson 1985, Møller and Lin 1986). It is still unclear whether the oxidation of NADH or NADPH is taking place on the cytoplasmic side, the apoplastic side, or on both sides of the plasma membrane. However, a more than 6-fold stimulation by the addition of Triton X-100 was recently reported for the NAD(P)H-dependent ferricyanide reductase (Buckhout and Hrubec 1986) indicating that, at least in this case, the NAD(P)H is oxidized on the cytoplasmic side of the plasma membrane. There are also various models to explain the possible connection of the plasma membrane redox activity with proton translocation (Lüttge and Clarkson 1985, Rubinstein and Stern 1986). Most reports show that H^+ -excretion is stimulated by extracytoplasmic e^- -acceptors, thus implicating either a pathway for H^+ -transport through the redox system itself, or suggesting that the redox activity effects the H^+ -translocating ATPase of the plant plasma membrane (Lüttge and Clarkson 1985, Rubinstein and Stern 1986). The former suggestion would imply an additional mechanism, besides H^+ -translocating ATPases, for driving active transport across the plant plasma membrane. The latter suggestion introduces a new perspective on how H^+ -translocating ATPases are functioning. Furthermore, superoxide and H_2O_2 production by the plasma membrane associated NAD(P)H redox system (Briskin et al. 1985, Møller and Bérczi 1985, 1986) may be biologically significant in defence against pathogens. Peroxidase activity (i.e., H_2O_2 production) is also known to be an activity necessary for lignification (Mäder and Amberg-Fisher 1982).

6.2. Blue-light photomorphogenesis

Several physiological responses in plants, such as proton extrusion in guard cells, are regulated by blue light (Zeiger 1983). A blue-light receptor, in conjunction with phytochrome, has also been shown to regulate gene expression in green leaves (Fluhr and Chua 1986). One possible

photoreceptor has been identified as a flavoprotein-cytochrome complex, and the cytochrome of this complex can be reduced by blue light, probably via a flavoprotein (for reviews, see Briggs and Ino 1983, Widell 1986). Spectral analysis of this plasma membrane bound b-type cytochrome (Widell et al. 1983) has revealed similarities with a cytochrome previously identified as cytochrome P-420 in microsomes of cauliflower inflorescences (Rich and Bendall 1975). Pure plasma membranes of this species showed CO-difference spectra and binding spectra of pyridine strongly indicating the presence of cytochrome P-450/420 (IV). Mild lithium dodecylsulfate polyacrylamide gel electrophoresis revealed a single heme-staining band at approximately 93 kDa. Plasma membranes of spinach and barley, on the other hand, both showed heme-staining bands at ca 40 kDa (Kjellbom and Larsson, unpublished results), similar to the molecular weights of mammalian liver cytochromes P-450 (West 1980). The high molecular weight for the heme-staining component of cauliflower plasma membranes does not exclude the possible involvement of cytochrome P-450 in this case. For instance, the component might be a dimer of cytochrome P-450, or the cytochrome could be associated with one or several polypeptides of the flavoprotein-cytochrome complex.

The presence of cytochrome P-450 in plant plasma membranes would explain the relative high specific activity of NADPH cytochrome c reductase (a putative ER marker) previously found in several highly pure plant plasma membrane fractions (I, Lundborg et al. 1981, Widell and Larsson 1983, Sandelius et al. 1986), since the native substrate for this reductase is cytochrome P-450 rather than cytochrome c. Moreover, NADPH-cytochrome c reductase (i.e., NADPH-cytochrome P-450 reductase) is a flavoprotein. Thus, the light-reducible b-type cytochrome, previously inferred to have a role in blue-light photomorphogenesis (Briggs and Ino 1983), might be a cytochrome P-450/420 associated with a NADPH-cytochrome P-450 reductase. Other possible components of this complex could be cytochrome b_5 and NADH cytochrome b_5 reductase. These are known constituents of cytochrome P-450-dependent mixed function oxidases in animal ER (Oshino 1982). Redox spectra of barley plasma membranes (I) and plasma membranes from corn coleoptiles (Leong et al. 1981) show split α -bands, which may indicate the presence of cytochrome b_5 . Mixed function oxidases are known to modify plant hormones (West 1980). Furthermore, differentiated growth responses to blue light are thought to be transmitted by plant hormones. Thus, we hypothesize that a mixed function oxidase in the plasma membrane, after activation by blue light (e.g., conversion of cytochrome P-420 to cytochrome P-450), modifies (activates or inactivates) plant hormones, for instance by hydroxylation (IV, Rich and Lamb 1977, Benveniste et al. 1978), thereby initiating the physiological response.

Some of the events described above may well be connected since, for instance, the swelling of guard cells is due to electrogenic proton pumping activated by blue-light (Assman et al. 1985). Furthermore, the NAD(P)H redox system of plant plasma membranes and the flavoprotein- b-type-cytochrome complex appear to have several common properties. Future research will reveal whether they represent two disparate e^- -transport chains or whether they actually are connected or even identical.

6.3. Polyglucan synthesis

There is convincing evidence for the involvement of the plant plasma membrane in the synthesis of cell surface glucans, and both 1,3- β -glucan synthetase and 1,4- β -glucan synthetase activities have been correlated with the plasma membrane (Ray 1977, 1979, Van Der Woude et al. 1974, see section 3.). Callose, mainly thought to consist of 1,3- β -linkages, is for instance known to be deposited on the sieve-plates of sieve-tubes terminating the phloem activity either permanently or seasonally. Furthermore, callose is involved in stigma papillae formation as a response to self-incompatible mating, in depositions at sites of fungal penetration (i.e., around invading haustoria), as well as in pollen maturation and pollen tube growth, and may also be deposited as a response to viruses (Bell 1981, Fincher and Stone 1981, Cooper 1982, Heiniger 1983, Griffing et al. 1986, Kauss and Jeblick 1986, Morrow and Lucas 1986). Glucan synthetase II (GS II) activity, the activity usually connected with callose synthesis, does not seem to have any major role in cell wall regeneration by protoplasts. Glucan synthetase I (GS I) activity, the activity inferred to produce mainly 1,4- β -linkages, increases when cells begin to incorporate glucose into the cell wall (Langebartels et al. 1981). Ray (1980) suggests that GS I activity is responsible for the synthesis of the hemicellulose backbone, xyloglucan, albeit other investigators only hold it responsible for the incorporation of xyloglucan into the cellulose network (Hayashi and Matsuda 1981). The possibility that GS I might be a cellulose synthetase proenzyme has also been proposed (Shore et al. 1975, Hayashi and Matsuda 1981). This proenzyme is thought to be converted into cellulose synthetase after reaching the plasma membrane. Thus, while one possible GS I function would be the participation in cell wall biosynthesis, GS II might be an inducible callose synthetase activity, activated by invading microorganisms, environmental stresses (e.g., temperature and water stress) and developmental processes. The increased capacity of cell-free extracts to synthesize 1,3- β -glycosidic linkages upon heat shock, with a concomitant increase in callose deposition, supports the idea of an inducible 1,3- β -glucan synthetase activity (Musolan et al. 1975).

Localized deposits of callose (i.e., papillae) upon fungal, viral, or bacterial infections may effect processes related to host-pathogen interactions such as feeding, flow of pathogen or endogenous toxins, and the rate of growth of the pathogen itself (Speakman and Lewis 1978, Aist et al. 1979, Skou 1982). An interesting example is the barley-powdery mildew host-pathogen system where resistant barley mutants, with resistance genes in the *ml-o* locus, show an early and massive callose formation (i.e., appositions around haustoria) irrespective of the powdery mildew culture applied. On the other hand, *ML-a* resistant varieties of barley do not show higher callose formation than their susceptible counterparts (Skou et al. 1984). Thus, the connection between the ability of an early formation of callose appositions around invading haustoria and a general and durable resistance is suggested. The high level of plasma membrane associated 1,3- β -glucan synthetase activity is a possible explanation for the broad and stable resistance of *ml-o* mutants towards powdery mildew. Since well defined barley mutants are available, this is a typical example where molecular biology techniques could be very helpful for the elucidation of membrane structure and function as well as for a better understanding of host-responses and host-pathogen interactions.

7. Summary of own results.

Hitherto I have presented data that I find significant in the area of isolation and characterization of plant plasma membranes irrespective of whether the achievements are my own or others. In order for the reader of this thesis to be able to more clearly discern my own contributions (to the unravelling of the plasma membrane structure and function) I will below summerize what is presented in papers I to IV.

In paper I we present an aqueous polymer two-phase partition procedure for the purification of plant plasma membrane vesicles from light-grown barley and spinach leaves. The procedure is reasonably fast (a few hours) and the required equipment is found in most laboratories. Analysis of marker enzyme activities and specific staining of the plasma membrane indicate a purity close to 100 %. The thylakoid membrane, the most abundant membrane in light-grown leaves, comprising as much as 90 % of total membranes, is effectively removed. By using low-temperature spectroscopy, we present evidence for the presence of a blue-light-reducible b-type cytochrome in the leaf plasma membrane, similar to the b-type cytochrome earlier identified in plasma membranes of dark grown tissues (Brain et al. 1977, Leong et al. 1981, Widell et al. 1983) and inferred to have a role in blue-light photomorphogenesis (Senger and Briggs 1981, Briggs and Iino 1983, Widell 1986). We also show that the plant plasma membrane has a low protein content compared to intracellular membranes. The plasma membranes of barley and spinach contain about 30 and 40 % (w/w) protein, respectively. The corresponding figures for spinach leaf mitochondrial and thylakoid membranes are 77 and 60 % (w/w), respectively (Gardeström et al. 1983, Quinn and Williams 1983). In addition we show the polypeptide composition of spinach and barley plasma membranes as revealed by SDS-PAGE. Several general similarities between spinach and barley plasma membranes are thereby displayed.

In paper II we show that plasma membrane vesicles, obtained by phase partition, are sealed and right side-out. This is shown by measuring the latency of membrane marker enzyme activities upon addition of a detergent. The marker enzymes used were the plasma membrane associated K^+ -stimulated, Mg^{2+} -dependent ATPase and glucan synthetase II. Both of these enzymes are likely to have their active sites at the cytoplasmic side of the plasma membrane. We suggest that the intactness and the sidedness of the plasma membrane vesicles obtained are due to the two-phase purification procedure. By using two-phase partition a relatively isoosmotic environment can be

maintained throughout the preparation procedure, and the vesicles are separated according to differences in surface properties. Thus, sealed plasma membrane vesicles of uniform sidedness are obtained. These results contrast to the leaky vesicles of unknown sidedness obtained by sucrose density gradient centrifugation (Hodges and Mills 1986).

In paper III we use the method described in paper I for preparing plasma membrane vesicles from roots and leaves of light-grown barley. The plasma membrane vesicles from the two organs have nearly identical polypeptide composition as revealed by SDS-PAGE, albeit with different relative amount of some of the polypeptides. Between ca 80 and 100 % of the K^+ -stimulated, Mg^{2+} -dependent ATPase activities were shown to be latent, again indicating that the vesicles were sealed and right side-out. Furthermore, the isoelectric points of the outer surface of the root and leaf plasma membrane vesicles were determined by cross-partition, and found to be similar and quite acidic - about pH 3.6. In contrast, the partition at the isoelectric point and the surface net charge density at pH 7.0, as determined by 9-aminoacridine fluorescence, differed significantly. In view of the striking similarities in polypeptide composition we suggest that the main functions of the plasma membrane are similar in roots and leaves, differing mainly in the balance between different processes. We also suggest that the large differences in surface net charge density, and hydrophobic/hydrophilic properties (as revealed by the different partition at the isoelectric point) may be due to larger differences in the lipid composition. Recent lipid analysis support this assumption (Rochester et al. 1986b). In Tab. 3 (section 5.5.) it can be seen that the relative amount of many of the major lipids varies considerably between root and leaf plasma membranes of barley, and there are even qualitative differences (the presence of acylated sterol glycosides in the root plasma membrane).

In paper IV we present evidence for the presence of cytochrome P-450/420 in plasma membranes of cauliflower inflorescences. This is indicated by CO-difference spectra and pyridine binding spectra. Mild lithium dodecylsulfate polyacrylamide gel electrophoresis shows a heme-staining band in the cauliflower plasma membrane fraction at ca 93 kDa. Since animal cytochromes P-450 have molecular weights in the range 40-50 kDa, we suggest that the 93 kDa band is a dimer. We have later shown that heme-staining bands of spinach and barley leaf plasma membranes have molecular weights of ca 40 kDa (Kjellbom et al. 1986a).

We suggest that the cytochrome P-450/420 is identical to the blue-light-reducible b-type cytochrome, inferred to be involved in blue-light photomorphogenesis (Senger and Briggs 1981, Briggs and Iino 1983), and that it is a subunit of a mixed-function oxidase. Since mixed-function oxidases are known to modify plant hormones (mainly by hydroxylation), the following model for the primary events of blue-light photomorphogenesis is proposed. When blue light is absorbed an inactive form of the mixed-function oxidase is transformed into an active one. The mixed-function oxidase then starts the physiological response by converting, for example, an inactive plant hormone into an active one. According to this model the specificity of the response should be offered by the cytochrome P-450 subunit. A high specificity in one of the steps is necessary since blue light has several different effects.

8. Epilogue

With the method of aqueous polymer two-phase partition highly pure plant plasma membrane vesicles are obtained. The vesicles have a defined sidedness (right side-out), they are sealed, and have so far been obtained from all species and organs tested, even from green leaves where the presence of thylakoid membranes offers a special challenge. Right side-out plasma membrane vesicles are very useful for many types of studies. A large number of studies have also been performed, including experiments aimed at elucidating the general structure and function of the membrane, as well as studies on discrete plasma membrane associated activities and proteins. A prerequisite for studying active transport across the membrane, however, is the access to inside-out plasma membrane vesicles, since the active site of the ATPase and probably also of the NAD(P)H-redox system are located on the cytoplasmic side of the membrane. Thus, to energize the vesicles and be able to measure active transport, inside-out vesicles are necessary. To prepare inside-out plasma membrane vesicles, either by inversion of right side-out vesicles, or by direct isolation from a microsomal fraction, would therefore represent another important step forward in plant plasma membrane research.

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Lund, in September, 1986.

Per Kjellbom

Till alla vänner som har undrat vad jag egentligen har sysslat med när ingen såg på:

Alla celler är omgivna av ett cellmembran, plasmamembranet. I växter sitter plasmamembranet innanför cellväggen och utgör cellernas egentliga avgränsning mot omvärlden. För att en växt skall kunna fortleva behövs ett ständigt utbyte av ämnen mellan växtens olika delar, mellan olika celler och mellan cellerna och omgivningen. Transporten av ämnen sker dels genom tubformade förbindelser mellan cellerna, men också genom direkt passage genom plasmamembranet. Hur denna sistnämnda transport går till vet vi mycket lite om, men på något sätt är den emellertid strikt reglerad av växtens och cellens behov av näringsämnen m.m.. Man vet mycket lite om växtplasmamembraner ty det har varit mycket svårt att isolera dem, d.v.s. avskilja dem från alla andra typer av membraner som finns i växtceller. Nu har emellertid jag och Christer Larsson, i samarbete med en hel del andra forskare, lyckats framställa rena preparationer av växtplasmamembraner från gröna växter. Vi kan nu således undersöka plasmamembraners uppbyggnad och funktion mer ingående, och det gör vi också.

Lund, i september, 1986

Per

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Preparation and polypeptide composition of chlorophyll-free plasma membranes from leaves of light-grown spinach and barley

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Chlorophyll-free preparations of plasma membranes from leaves of barley (*Hordeum vulgare* L. cv. Kristina) and spinach (*Spinacia oleracea* L. cv. Viking II) were obtained by partition in an aqueous dextran-polyethylene glycol two-phase system. Glucan synthetase II (EC 2.4.1.34), a marker for the plasma membrane, was highly enriched in both preparations. Silicotungstic acid, a specific stain for the plasma membrane, indicated a purity close to 100% for the barley preparation. Both plasma membrane preparations contained a light-reducible *b*-cytochrome, as shown by low temperature spectroscopy. The plasma membranes had a low protein content compared to the bulk of intracellular membranes. The polypeptide composition of the barley and spinach plasma membranes showed striking similarities, with the most prominent polypeptides in the 49–58 kdalton region, and some further prominent bands in the 30 kdalton region. Some high molecular weight polypeptides in the 73–110 kdalton region were also typical for the plasma membranes compared to the microsomal fractions.

Additional key words – *Hordeum vulgare*, light-reducible *b*-cytochrome, phase partition, *Spinacia oleracea*.

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Introduction

The plasma membrane is the outer, permeability barrier of the plant cell. This imposes several important functions on the plasma membrane, such as transport of ions and photosynthetic products, hormone binding, cell wall synthesis and interactions with pathogens. Some of these functions are largely restricted to certain organs of the plant. This is the case with export of carbon assimilation products, an event restricted to the photosynthesizing leaf cells. Similarly, certain plant pathogen interactions are confined to the leaves. The study of the role of the plasma membrane in these events therefore requires the isolation of plasma membranes from green leaves. This is very difficult to achieve with the most commonly used methods for plasma membrane isolation, namely different types of density gradient centrifugation, since plasma membranes and fragments of the

thylakoid membrane band at similar densities. As an alternative to gradient centrifugation, partition in aqueous dextran-polyethylene glycol two-phase systems was introduced in the purification of plasma membranes (Lundborg et al. 1981, Widell and Larsson 1981). This method separates membrane vesicles according to their surface properties rather than according to their size and density.

In the present study we have obtained chlorophyll-free preparations of plasma membranes from green leaves of both a mono- and a dicotyledon (barley and spinach) using phase partition. The plasma membranes have been characterized with respect to different markers, and with respect to the polypeptide composition as determined by sodium dodecylsulphate polyacrylamide gel electrophoresis.

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Abbreviations – α -CF₁, β -CF₁, the α - and β -subunits, respectively of the coupling factor (CF₁) of chloroplast thylakoids; LHCP, the apolipoprotein of the light-harvesting chlorophyll-protein complex; L-carb, the large subunit of ribulose-1,5-bisphosphate carboxylase; L₁, the lower phase plus interface left in tube 1 (see Fig. 1D) containing intracellular membranes; MF, microsomal fraction; U₁, U₁, U₂, U₃, U₃, upper phases containing plasma membranes of increasing purity (see Fig. 1).

Materials and methods

Preparation of plasma membranes. Spinach (*Spinacia oleracea* L. cv. Viking II) was grown in a nutrient solution according to Siegenthaler and Depéry (1976) except that the Fe-concentration was doubled. Barley (*Hordeum vulgare* L. cv. Kristina) seedlings were grown in vermiculite with the same nutrient solution as above. The temperature in the growth chamber varied between 17°C in the dark and 23°C during the light period, which was 12 h per day. The light was provided by fluorescent tubes (Power groove, cool white, General Electric, U.S.A.) and dysprosium bulbs (HQI-E 400W/DH, Osram) which together gave a photon fluence rate of about 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Eight-day-old barley seedlings and eight-week-old spinach leaves were used for the preparations. All procedures were carried out at 4°C. The leaves (125 g) were cut into pieces and placed in a vacuum flask with 275 ml of a slightly hypertonic medium consisting of 0.5 M sucrose, 50 mM Hepes-KOH, pH 7.5, 5 mM ascorbic acid, 3.6 mM cysteine and 0.6% polyvinylpyrrolidone; ascorbic acid and cysteine were added as solids just before use. Partial plasmolysis was facilitated by vacuum-infiltration of the medium. The material was transferred to a Braun homogenizer, equipped with razor blades instead of the ordinary knives (Kannangara et al. 1977), and homogenized 4 times for 25 s. The homogenate was filtered through a 240 μm nylon cloth and centrifuged at 10 000 g for 10 min. A microsomal pellet was obtained from the supernatant by centrifugation at 50 000 g for 30 min. This pellet was suspended to a total volume of 10 ml in 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8; and 9 g of the suspension (50–60 mg protein) was

added to a 27 g phase mixture to give a 36 g two-phase system with a final composition of 6.2% (w/w) Dextran T 500, 6.2% (w/w) polyethylene glycol 3350 (earlier designated 4000), 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8. The phase system was thoroughly mixed and phase settling was accelerated by centrifugation in a swinging bucket centrifuge at 1 500 g for 3 min. Further processing was according to Fig. 1, except that a batch procedure of five steps was needed to obtain a chlorophyll-free preparation of plasma membranes from barley. The two upper phases (U₃ and U₃) were pooled, diluted, and the material recovered by centrifugation at 100 000 g for 30 min. The lower phase plus interface left in tube 1 (L₁), and the part of the microsomal fraction which was saved, were diluted with an appropriate medium and the membranes recovered by centrifugation. The pellets were suspended in the same medium and kept on ice, or were frozen in liquid nitrogen.

Protein was determined with a modified Bearden (1978) procedure, using Triton X-100 to solubilize membrane proteins. The fractions were first exposed to a high (about 0.1%, w/v) Triton concentration, and then water and reagents were added to bring the Triton concentration to 0.01%. This final concentration of Triton X-100 did not disturb the assay.

Chlorophyll was determined according to Arnon (1949).

Elementary analysis of C, H and N were performed with a Carlo Erba CHN-Analyzer.

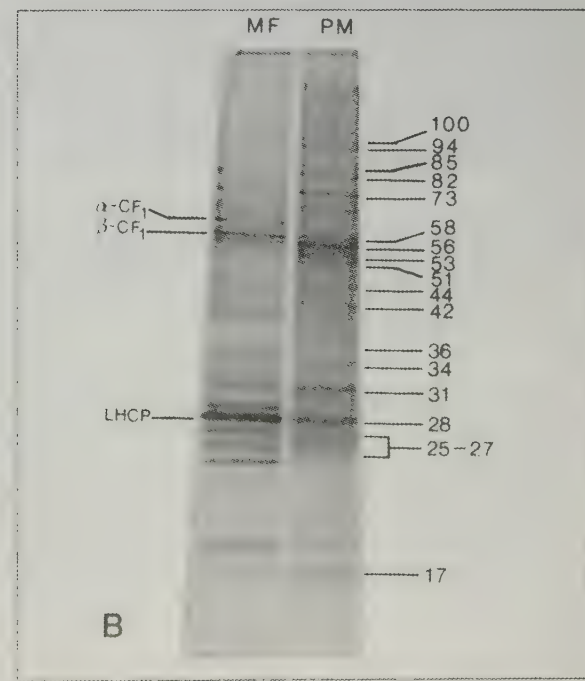
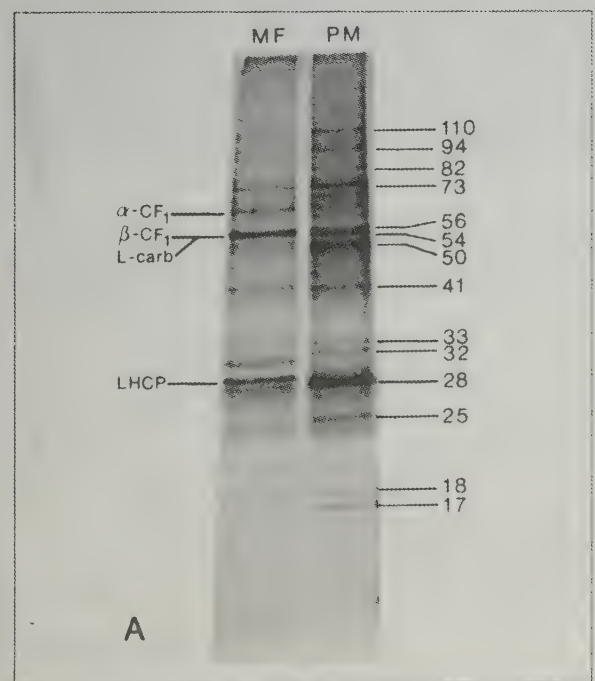
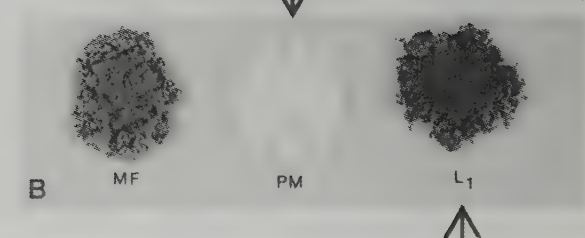
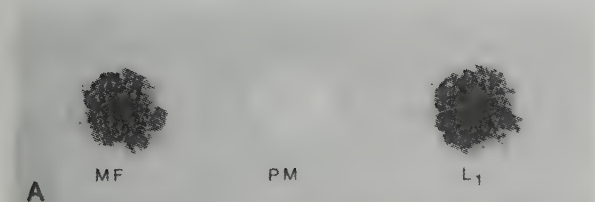
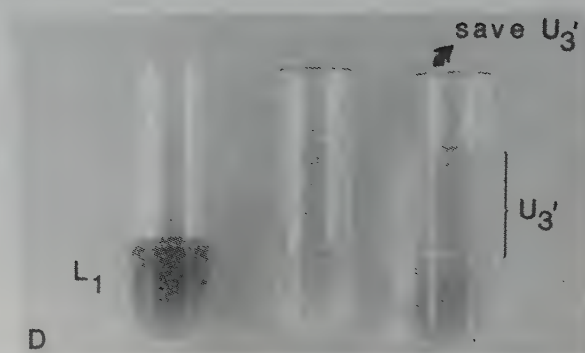
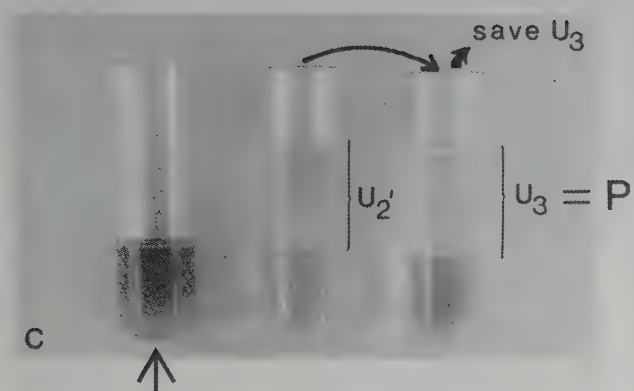
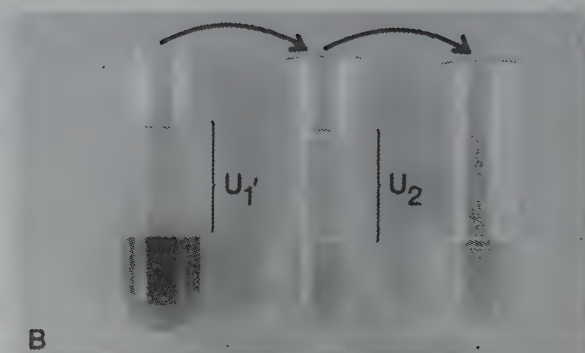
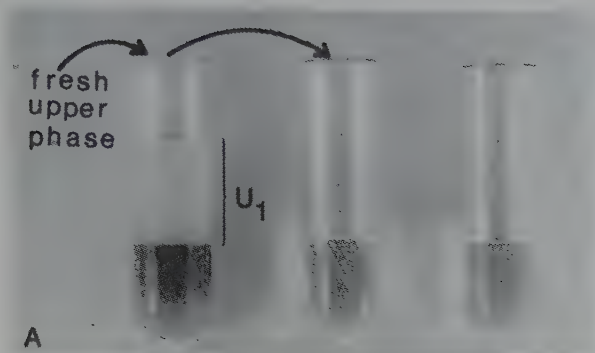
Cytochrome c oxidase (EC 1.9.3.1) and **NADPH-cytochrome c reductase** (EC 1.6.2.5) were determined essentially according to Hodges and Leonard (1974), using 0.01% (w/v) Triton X-100 in the assay medium.

Glucan synthetase II (EC 2.4.1.34) was assayed according to Ray (1979), with the modification that we used 0.15 M instead of 0.3 M cellobiose as primer.

Fig. 1. Separation of plasma membranes and intracellular membranes by a batch procedure (A-D). The microsomal fraction, obtained from spinach leaves and suspended in the medium used in the phase system, was added to a preweighed phase mixture. The phase system was well mixed by 20 to 30 inversions of the tube, and phase separation was facilitated by a low-speed centrifugation in a swinging-bucket centrifuge for a few min. Then, 90% of the upper phase (U₁) was removed, and re-partitioned twice (B, C) with fresh lower phase to increase the purity, giving U₃. To increase the yield of plasma membranes, the lower phases were re-extracted, in sequence (B-D), with a fresh upper phase, giving U₃. The upper phases (U₃ and U₃) were pooled, diluted several-fold, and the plasma membranes collected by ultracentrifugation. Fresh upper and lower phases were obtained from a bulk phase system (of e.g., 300 g) of identical composition prepared in a separating funnel.

Fig. 2. Freeze-dried material from leaves of barley (A) and spinach (B), obtained by the batch-procedure outlined in Fig. 1. MF, microsomal fraction; PM, plasma membranes (U₃ + U₃); L₁, intracellular membranes recovered from the lower phase plus interface (Fig. 1).

Fig. 7. Sodium dodecylsulphate polyacrylamide gel electrophoresis of the plasma membrane (PM) and microsomal (MF) fractions obtained from leaves of spinach (A) and barley (B). The molecular weights (kdalton) of prominent plasma membrane polypeptides are indicated. The gels were silver stained (Guevara et al. 1982). Abbreviations, see Fig. 5.



Low temperature spectroscopy was performed using an Aminco DW-2 spectrophotometer equipped with a low temperature accessory. Blue light-reduced minus dark spectra were obtained using a Xenon lamp and a monochromator. The procedure described by Widell et al. (1983) was followed.

Electron microscopy. Specific staining of plasma membranes with silicotungstic acid was essentially according to Roland (1978). Uranyl acetate staining was according to standard procedures.

Sodium dodecylsulphate polyacrylamide gel electrophoresis on gradient gels [$T = 12\text{--}20\%$, $C = 2.7\%$, according to the nomenclature of Hjertén (1962); gel dimension $140 \times 80 \times 2.7$ mm] was according to Laemmli (1970) using a circulating "running" buffer. During the passage through the stacking gel, a current of 5 mA gel^{-1} was applied, and during the rest of the electrophoresis 7.5 mA gel^{-1} . Total electrophoresis time was 20 h. The positions of major chloroplast thylakoid polypeptides were determined by co-electrophoresis with isolated thylakoids.

Silver staining of gels was essentially according to Guevara et al. (1982).

Chemicals. Dextran T 500 (average M_r 500 000) was obtained from Pharmacia Fine Chemicals, Uppsala, Swe-

den. Polyethylene glycol 3350 (average M_r 3350) was obtained from Union Carbide, N.Y., USA.

Results

The purification of plasma membranes was followed via the distribution of different membrane markers (Tab. 1). A large part of the plasma membrane marker, glucan synthetase II, was recovered in the pooled upper phases (U_3 and U_3'), indicating a recovery of plasma membranes of about 40 and 60% for barley and spinach, respectively. These figures are probably underestimates since glucan synthetase II activity is likely to be associated also with intracellular membranes (Ray 1979); and relatively large amounts of glucan synthetase II were also found in the intracellular membrane fractions (L_1). The markers for chloroplasts (chlorophyll), mitochondria (cytochrome *c* oxidase) and endoplasmic reticulum (NADPH-cytochrome *c* reductase) were almost completely distributed to the lower phase and recovered in fraction L_1 . No chlorophyll was detected in the plasma membrane preparations by standard procedures and the material was white (Fig. 2). It is also obvious from photographs of the batch procedure (Fig. 1) that chlorophyll was almost completely distributed to the lower phase.

The enrichment of glucan synthetase II in the plasma membrane fraction was several-fold with both species, whereas the activity was depleted in the L_1 fraction con-

Tab. 1. Total protein, chlorophyll and marker enzyme activities in the plasma membrane ($U_3 + U_3'$) and the intracellular membrane (L_1) fractions obtained by phase partition of microsomal fractions (MF) from leaves of barley and spinach. Data are the mean $\pm$ SD of four (barley), and three (spinach), independent experiments, respectively. ND, not detectable.

Species	Fraction	Protein mg	Chlorophyll mg	Glucan synthetase II nmol min^{-1}	Cytochrome <i>c</i> oxidase nmol min^{-1}	NADPH-cytochrome <i>c</i> reductase nmol min^{-1}
Barley	MF	58 $\pm$ 12	6.7 $\pm$ 0.7	12.5 $\pm$ 4.2	7400 $\pm$ 1200	470 $\pm$ 140
	$U_3 + U_3'$	1.3 $\pm$ 0.2	ND	4.7 $\pm$ 1.5	42 $\pm$ 13	6.5 $\pm$ 0.6
	L_1	28 $\pm$ 9	3.1 $\pm$ 0.3	5.4 $\pm$ 2.1	5100 $\pm$ 1100	280 $\pm$ 58
Spinach	MF	49 $\pm$ 2	4.0 $\pm$ 0.2	63 $\pm$ 7	19000 $\pm$ 4600	610 $\pm$ 130
	$U_3 + U_3'$	4.0 $\pm$ 0.9	ND	37 $\pm$ 7	92 $\pm$ 51	29 $\pm$ 8
	L_1	22 $\pm$ 2	2.8 $\pm$ 0.2	11 $\pm$ 2	14200 $\pm$ 3200	360 $\pm$ 47

Tab. 2. Specific marker enzyme activities in the plasma membrane ($U_3 + U_3'$) and the intracellular membrane (L_1) fractions obtained by phase partition of microsomal fractions (MF) from leaves of barley and spinach. Data are the mean $\pm$ SD of four (barley), and three (spinach), independent experiments, respectively.

Species	Fraction	Glucan synthetase II nmol $\text{mg}^{-1} \text{min}^{-1}$	Cytochrome <i>c</i> oxidase nmol $\text{mg}^{-1} \text{min}^{-1}$	NADPH-cytochrome <i>c</i> reductase nmol $\text{mg}^{-1} \text{min}^{-1}$
Barley	MF	0.21 $\pm$ 0.09	128 $\pm$ 9	7.9 $\pm$ 0.9
	$U_3 + U_3'$	3.53 $\pm$ 0.27	31 $\pm$ 13	4.8 $\pm$ 0.9
	L_1	0.19 $\pm$ 0.12	181 $\pm$ 36	9.9 $\pm$ 1.6
Spinach	MF	1.29 $\pm$ 0.25	389 $\pm$ 33	12.4 $\pm$ 2.2
	$U_3 + U_3'$	9.39 $\pm$ 2.24	23 $\pm$ 10	7.2 $\pm$ 1.5
	L_1	0.53 $\pm$ 0.03	661 $\pm$ 79	16.9 $\pm$ 1.9

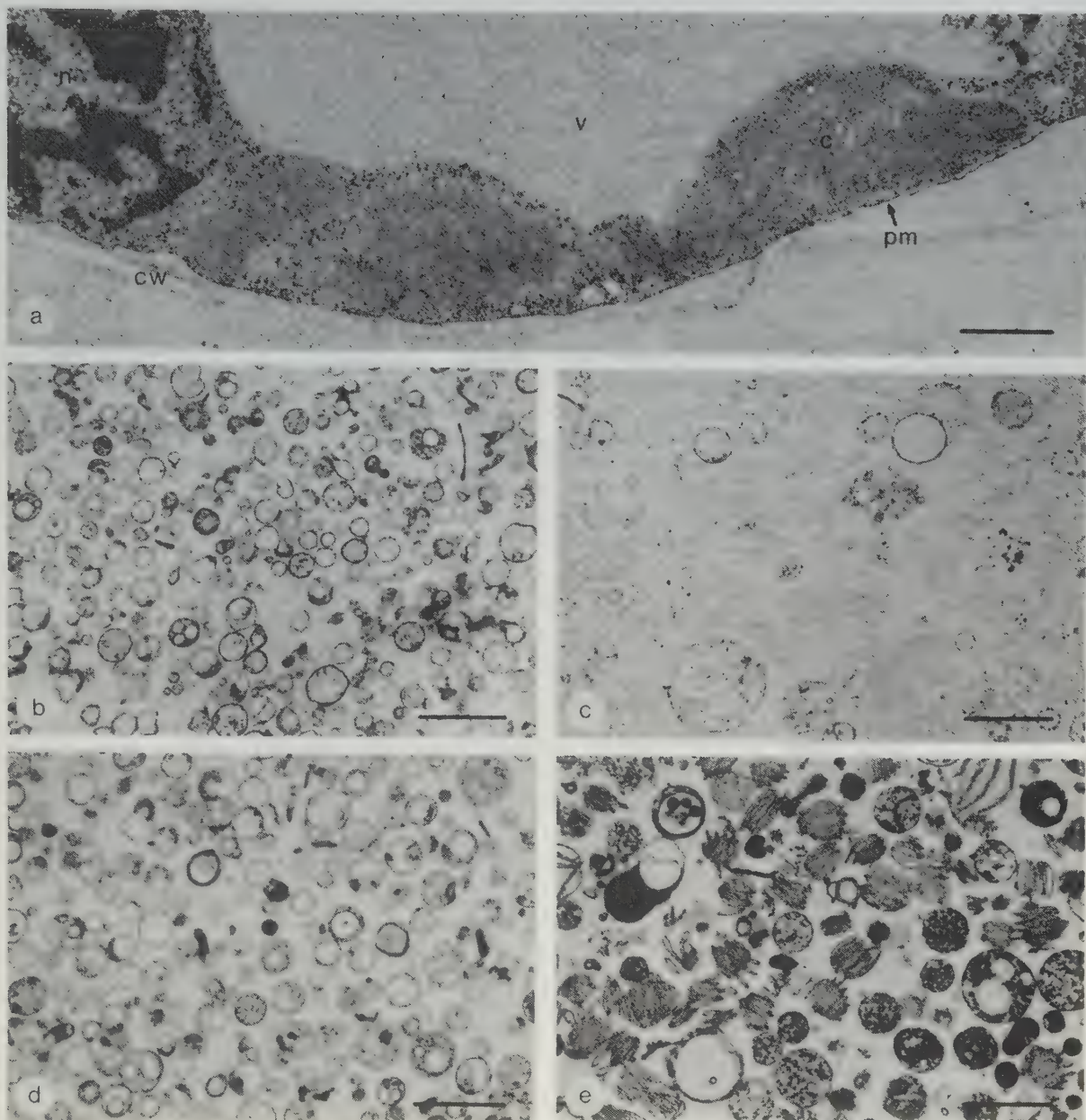


Fig. 3. Silicotungstic acid staining (Roland 1978) of a, a section of a barley leaf; b, the plasma membrane fraction; and c, the microsomal fraction used as the starting material. Parts d and e show the plasma membrane and microsomal fractions, respectively, stained with uranyl acetate for comparison. Note the specificity of the silicotungstic acid stain for the plasma membrane. Abbreviations: c, chloroplast; cw, cell wall; n, nucleus; pm, plasma membrane; v, vacuole. Bar 1 μ m in all part figures.

taining intracellular membranes (Tab. 2). Glucan synthetase II was enriched 16-fold in barley and 7-fold in spinach plasma membranes compared to the microsomal fractions used as starting material.

A relatively high specific activity of the marker for endoplasmic reticulum, NADPH-cytochrome *c* reductase, was found in the plasma membrane preparations

(Tab. 2), although the bulk of the activity was distributed to the lower phase (Tab. 1).

Electron micrographs (Fig. 3 b,d) of the plasma membrane fraction from barley shows closed structures, indicating vesicles with diameters of 0.1 to 0.5 μ m. Using a specific stain for the plasma membrane – silicotungstic acid (Roland, 1978) – virtually all vesicles in the

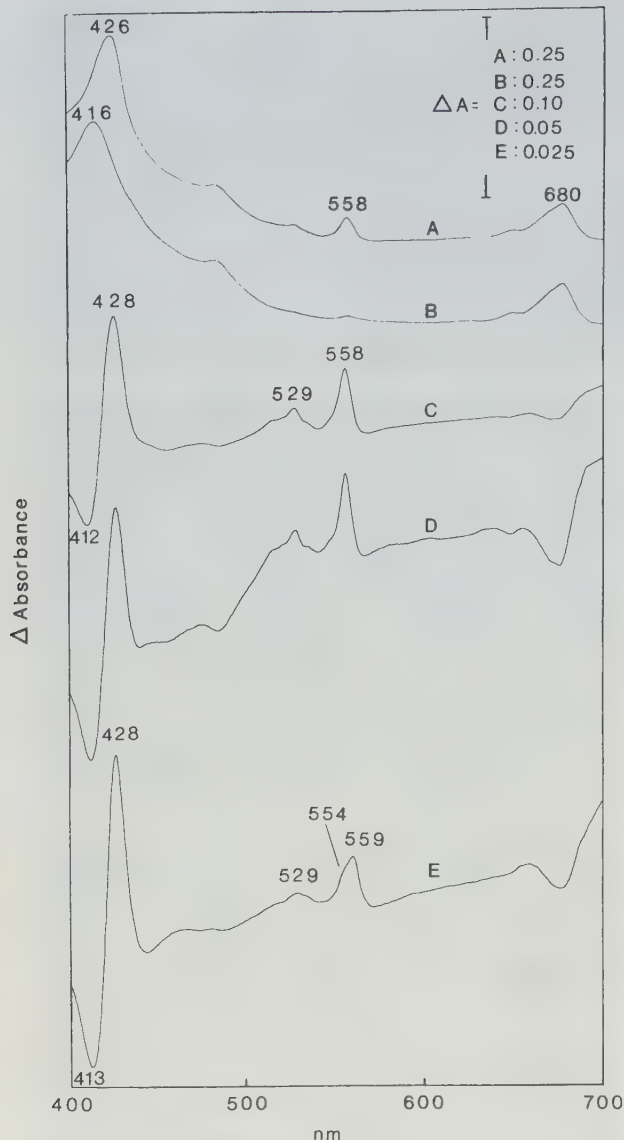


Fig. 4. Low temperature (77 K) spectra of the plasma membrane fraction ($U + U_3$) from leaves of spinach (A–D) and barley (E). A, dithionite reduced; B, oxidized; C, dithionite-reduced minus oxidized; D, blue light-reduced minus oxidized (dark); E, dithionite-reduced minus oxidized (barley).

plasma membrane fraction were well stained (Fig. 3 b), whereas only a few vesicles were well stained in the microsomal fraction (Fig. 3 c).

Low temperature spectroscopy of plasma membrane preparations from spinach and barley (Fig. 4) revealed that both contained a cytochrome. This cytochrome could in both species be reduced either by dithionite or by blue light (not shown for barley), and is thus similar to the light-reducible *b*-cytochrome described earlier for plasma membranes from non-green tissues (Brain et al. 1977, Leong et al. 1981, Widell et al. 1983). The α -band appeared at 558 nm for spinach and at 559 nm for bar-

ley, with a shoulder at approximately 554 nm for the latter species. The β -band appeared at the same wavelength, 529 nm, for both species. In the Soret region the maxima appeared at 428 nm, and the minimum at 412 nm for spinach and at 413 nm for barley. (Note, that when compared with room temperature spectra, the wavelengths given here should be increased by 3 nm.) A small chlorophyll peak was visible around 680 nm, although no chlorophyll was detected by standard methods.

The plasma membranes of barley and spinach contained about 30 and 40% (w/w) protein, respectively, as revealed by elementary analysis (Tab. 3), assuming that all nitrogen is present in proteins and that proteins on an average contain 16% nitrogen. The corresponding figures for the microsomal fractions were 51 and 55%, respectively. The latter value may be compared with values in the literature for the spinach thylakoid membrane of around 60% (Quinn and Williams 1983) and for spinach leaf mitochondrial membranes of 77% (w/w) protein (Gardeström et al. 1983). Thus, the plasma membrane seems to contain relatively little protein. However, the polypeptide composition of the plasma membrane is still very complex, and for both species more than 40 bands are detected after sodium dodecylsulphate polyacrylamide gel electrophoresis on gradient gels (Figs 5, 6 and 7). For the spinach plasma membrane, Coomassie staining shows several prominent polypeptides as indicated in Fig. 5. Some of these polypeptides are almost fully depleted from the intracellular membrane fraction (L_1), such as the 110, 94, 82, 73, 59, 56, 51 and 49 kdalton polypeptides. With silver staining (Fig. 7 A) approximately the same polypeptide pattern is discernable for the spinach plasma membrane, but some of the bands which are heavily stained with the Coomassie stain are not so heavily stained with silver, and vice versa; e.g. the 28, 25 and 17 kdalton polypeptides are relatively better stained with silver than with Coomassie blue. These differences in specificity, between Coomassie and silver staining, are inherent in the different techniques (Guevara et al. 1982). The polypeptide patterns of the barley plasma mem-

Tab. 3. Elementary (N, C, H) analysis of plasma membrane fractions ($U_3 + U_3$) obtained by phase partition of microsomal fractions (MF) from leaves of barley and spinach. Pooled and freeze-dried fractions from more than 10 independent preparations were analysed. The data are given as % of dry weight. The percentage of protein is calculated on the assumption that proteins on average contain 16% nitrogen and that all nitrogen is in proteins.

Species	Fractions	N	C	H	Protein
Barley	MF	8.1	58.0	8.4	51
	$U_3 + U_3$	4.7	54.9	8.3	30
Spinach	MF	8.8	58.8	8.3	55
	$U_3 + U_3$	6.4	58.6	8.6	40

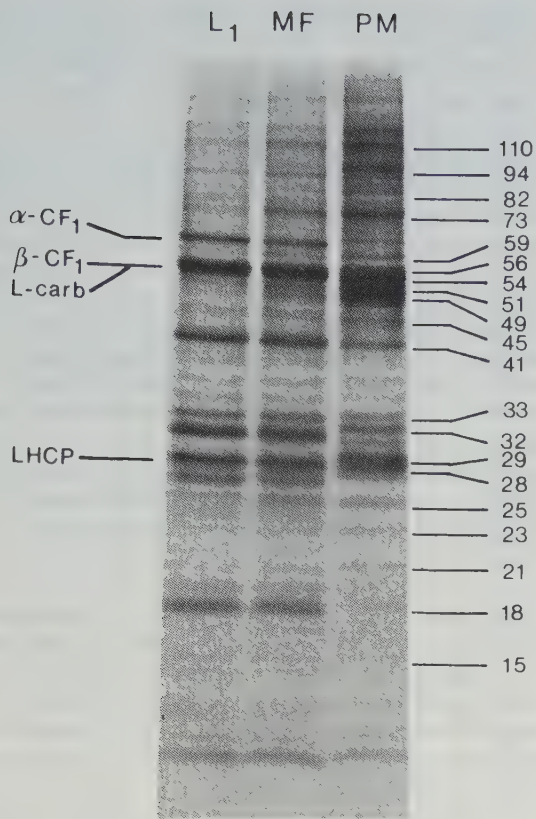


Fig. 5. Sodium dodecylsulphate polyacrylamide gel electrophoresis of the plasma membrane (PM) fraction from spinach leaves. The polypeptide patterns of the microsomal fraction (MF) used as the starting material, and the intracellular membrane fraction recovered from the lower phase plus interface (L_1 , compare Fig. 1) are shown for comparison. The molecular weights (kdalton) of prominent plasma membrane polypeptides are indicated. Note the depletion of plasma membrane polypeptides in the intracellular membrane fraction (L_1). The gel was stained with Coomassie brilliant blue. The positions of some major thylakoid polypeptides, namely the α - and β -subunits of the coupling factor (α -CF₁, β -CF₁), the large subunit of the ribulose-1,5-bisphosphate carboxylase (L-carb) and the apopolypeptide of the light-harvesting chlorophyll-protein complex (LHCP), are indicated.

brane and microsomal fraction are shown in Figs 6 and 7 B. Prominent plasma membrane bands are indicated. As in the case of spinach, several polypeptides are highly enriched in the plasma membrane fraction compared to the microsomal fraction, such as the 100, 94, 85, 56, 53, 51, 45–44, 42, 36 and 31 kdalton polypeptides. Comparing the polypeptide patterns of barley and spinach plasma membranes, it is evident that several of the prominent polypeptides have very similar molecular weights, e.g. the 94, 82, 73, 56, 54–53, 51–50, 45, 42–41, 34–32 and 29–28 kdalton polypeptides. Thus, although derived from two not very closely related species, the polypeptide patterns of the plasma membranes show great similarities.

Discussion

The aims of this work were to prepare pure plasma membranes from light-grown barley and spinach leaves, and to characterize the plasma membranes biochemically, in particular with respect to the polypeptide composition.

The plasma membrane constitutes a minor part of the total amount of membrane in the cell. In light-grown leaves the chloroplast thylakoid membrane makes up the bulk of the membrane material, and only a few % is plasma membrane. Preparation of plasma membranes from green leaves therefore demands a method capable of separating plasma membranes from thylakoid membranes with high efficiency. As shown in previous studies using dextran-polyethylene glycol two-phase systems and non-green material (for reviews, see Larsson 1983, 1985) conditions may be found where the plasma membranes partition strongly in the polyethylene glycol-rich upper phase and intracellular membranes partition at the interface or in the lower phase. Similarly, it was

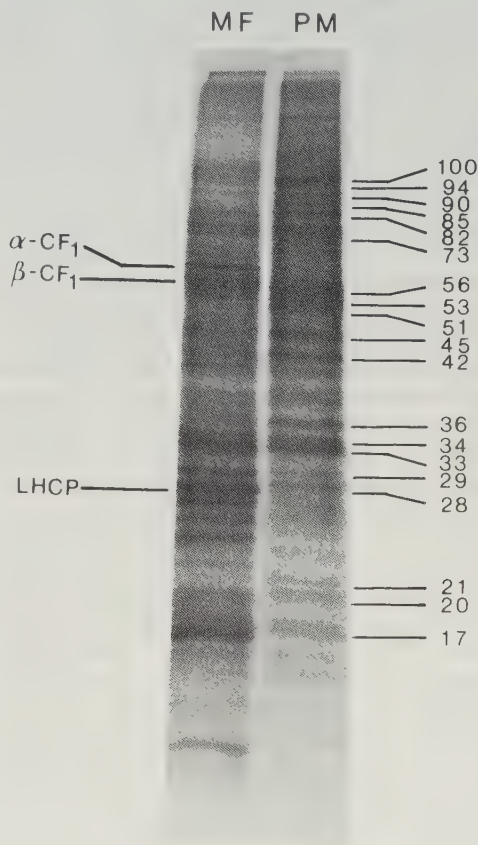


Fig. 6. Sodium dodecylsulphate polyacrylamide gel electrophoresis of the plasma membrane (PM) and microsomal (MF) fractions obtained from barley leaves. The molecular weights (kdalton) of prominent plasma membrane polypeptides are indicated. The gel was stained with Coomassie brilliant blue. Abbreviations, see Fig. 5.

shown earlier in studies with multiorganelle complexes surrounded by plasma membrane (Larsson et al. 1971, Larsson and Andersson 1979), and in studies with protoplasts (Kanai and Edwards 1973, Hallberg and Larsson 1981), that the plasma membrane has surface properties which permits its separation from thylakoid membranes by phase partition. As shown in a preliminary report (Kjellbom and Larsson 1984) and in the present study, an almost complete separation of plasma membranes and thylakoid membranes may conveniently be achieved in a two-phase system with carefully adjusted polymer and salt concentrations to optimize plasma membrane recovery and purity. The plasma membranes obtained were chlorophyll-free and also very pure with respect to contamination by mitochondria (Tabs 1 and 2). Staining of thin sections with silicotungstic acid, a specific stain for the plasma membrane (Roland 1978), showed practically only well-stained vesicles in the plasma membrane preparation from barley leaves (Fig. 3), suggesting a purity close to 100%.

However, a relatively high specific activity of cytochrome *c* reductase was found in both plasma membrane preparations (Tab. 2), in agreement with results obtained previously with plasma membranes from non-green material (Lundborg et al. 1981, Widell and Larsson 1983). We have earlier proposed (Widell et al. 1982) that this cytochrome *c* reductase activity could be due to the enclosure of small vesicles of endoplasmic reticulum within larger plasma membrane vesicles. This hypothesis was tested by sonication of isolated plasma membrane vesicles and subsequent re-partitioning in a two-phase system. By this treatment the vesicles of endoplasmic reticulum should be released if present, and subsequently separated from the plasma membranes upon re-partitioning. However, the specific activity of NADPH-cytochrome *c* reductase was not decreased in the plasma membranes recovered from the upper phase after re-partitioning (data not shown), indicating that the activity is truly plasma membrane-bound. Recent experiments show that plasma membranes from cauliflower inflorescences contain cytochrome P-450/420, as judged from CO-difference spectra (C. Schelin, P. Kjellbom, S. Widell and C. Larsson, unpublished results). The presence of cyt P-450/420 in the plasma membrane would explain the presence of NADPH-cytochrome *c* reductase activity in the plasma membrane preparations, since cytochrome P-450/420 is associated with NADPH-cytochrome *c* reductase activity (e.g., Strobel and Dignam 1983). Moreover, it was recently suggested (Widell and Larsson 1984) that the plasma membrane-bound light-reducible *b*-cytochrome, inferred to have a role in photomorphogenesis (Senger and Briggs 1983), is a cytochrome P-450/420. This light-reducible *b*-cytochrome has earlier been found in plasma membrane preparations from different non-green material (Brain et al. 1977, Widell and Larsson 1983), and is also present in plasma membranes from green leaves (Fig. 4). Thus, the NADPH-cytochrome *c* reductase ac-

tivity present in the plasma membrane preparations is probably not due to contamination by endoplasmic reticulum, but is a true property of the plasma membrane, and probably associated with the light-reducible *b*-cytochrome.

The light-reducible *b*-cytochrome of spinach plasma membrane has a rather symmetrical α -band at 558 nm, similar to the corresponding cytochrome of the cauliflower plasma membrane (peak at 556 nm, Widell et al. 1983). The barley plasma membrane, on the other hand, shows an α -band at 559 nm with a prominent shoulder at about 554 nm, similar to the plasma membrane of corn coleoptiles (peak at 555 nm, shoulder at 550 nm, Leong et al. 1981). The α -bands observed with barley and corn plasma membranes could represent two different cytochromes, but may alternatively be due to one cytochrome showing a split α -band. Cytochrome P-450/420, which shows two bands in CO-difference spectra, might be a candidate for a cytochrome with a split α -band.

As revealed by analysis of the N, C and H contents (Tab. 3) the plasma membranes from both spinach and barley have a low protein content compared to the bulk of intracellular membranes in the microsomal fractions. That the plasma membrane is a lipid-rich membrane compared to the intracellular membranes is supported by a preliminary lipid analysis on oat root plasma membranes (Lundborg et al. 1983).

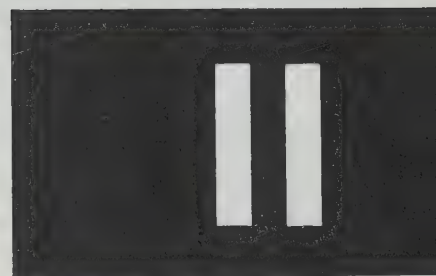
The polypeptide patterns of plasma membranes from barley and spinach show several striking similarities, which clearly distinguish the plasma membranes from their corresponding microsomal fraction. The most prominent bands are for both species found in the 49–58 kdalton region, where at least four closely spaced bands are discernable (Figs 5, 6 and 7). Further similarities are found in the 30 kdalton region with several prominent bands. Some high molecular weight polypeptides in the 73–110 kdalton region are also typical for the plasma membranes compared to the microsomal fractions. So far, only one of the plasma membrane polypeptides has been identified, namely the subunit of the K^+ -stimulated Mg^{2+} -ATPase which undergoes reversible phosphorylation. This subunit was found to have a molecular weight of about 100 kdalton in both oat root (Vara and Serrano 1983), and red beet (Briskin and Poole 1983). However, for a better understanding of the functions of the plasma membrane the identification of further plasma membrane polypeptides is required. These polypeptides should include subunits of several different transport proteins, such as a sucrose translocator, inferred to be present in the plasma membrane of photosynthetically active cells (Huber and Moreland 1981), as well as the light-reducible *b*-cytochrome, with a possible role in blue light photomorphogenesis (Senger and Briggs 1983). Furthermore, protein receptors involved in host-pathogen interactions, such as plant resistance, are thought to reside in the plasma membrane (Albersheim and Andersson-Prouty 1975). The identification of

such receptors should be invaluable for future breeding for resistance.

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Sidedness of plant plasma membrane vesicles purified by partitioning in aqueous two-phase systems

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Preparations of plant plasma membrane vesicles were obtained by partition in dextran–polyethylene glycol two-phase systems. By this procedure particles are separated according to their surface properties, and an iso-osmotic environment is maintained throughout. The vesicles thus produced are right-side-out and sealed, as measured by enzyme [(K⁺ + Mg²⁺)-ATPase and glucan synthetase II] latency on addition of Triton X-100.

<i>Plant plasma membrane vesicle</i>	<i>Sidedness</i>	<i>Enzyme latency</i>	<i>K⁺-stimulated Mg²⁺-dependent ATPase</i>
	<i>Glucan synthetase II</i>	<i>Two-phase system</i>	

1. INTRODUCTION

Isolated plasma membrane vesicles are important material for studies on biological events associated with this membrane. However, for a correct interpretation of results on transport, phytochrome binding, hormone binding etc., it is important to use preparations of plasma membranes with a defined sidedness. The ideal situation, of course, would be to have pure preparations of both right-side-out and inside-out vesicles, respectively. Such preparations can be obtained from erythrocyte and chloroplast thylakoid membranes, and have been of great importance for the analysis of the transverse asymmetry of these membranes (review [1] and [2], respectively). With both erythrocytes and chloroplast thylakoids, membrane preparations of opposite sidedness are obtained by partition in aqueous dextran–polyethylene glycol two-phase systems. In fact, this method is extremely well suited for the preparation of membrane vesicles which are homogeneous with respect to sidedness, since it separates particles

with respect to surface properties [3] rather than size and density, as does centrifugation. We have prepared plasma membranes from several plant species using two-phase systems [4,5], and these preparations may therefore be expected to contain vesicles of uniform sidedness. These plasma membrane vesicles may furthermore be expected to be right-side-out, since they partition similarly to intact protoplasts, i.e., they have a high affinity for the polyethylene glycol-rich upper phase [6]. To test this hypothesis, we have studied the latency of K⁺-stimulated Mg²⁺-dependent ATPase and glucan synthetase II on addition of Triton X-100. These two enzymes are known to be localized in the plasma membrane [7] and the ATPase has generally been assumed to have its ATP-binding site on the cytoplasmic side [7], while results on the UDP-glucose-binding site of glucan synthetase II are conflicting [8,9]. Our results, obtained with cauliflower inflorescences and oat roots, suggest that plant plasma membranes collected from the upper phase after purification by phase partition are mainly sealed right-side-out vesicles.

2. MATERIALS AND METHODS

2.1. Plant material

Oats (*Avena sativa*, L. cv. Brighton) were grown for 7 days at 18°C in darkness [5]. Cauliflower inflorescences (*Brassica oleracea* L.) were obtained at the local market.

2.2. Plasma membranes

Plasma membranes were purified from microsomal fractions of oat roots (10000–30000 × g fraction) and cauliflower inflorescences (10000–50000 × g fraction) by partitioning in an aqueous polymer two-phase system [4,5]. To avoid pelleting the material after phase partitioning, to keep the plasma membrane vesicles as intact as possible, we modified the composition of the phase system to allow for measurements of (K⁺ + Mg²⁺)-ATPase activity on aliquots withdrawn directly from the upper phase. Thus, potassium phosphate was exchanged for Tris-SO₄, and a phase system of the following composition was used: 6.5% (w/w) dextran T 500, 6.5% (w/w) polyethylene glycol 4000, 0.25 M sucrose, 2 mM Cl-Tris, 5 mM SO₄-Tris (pH 7.8).

2.3. K⁺-stimulated Mg²⁺-dependent ATPase

K⁺-stimulated Mg²⁺-dependent ATPase was determined essentially as in [10] in an iso-osmotic medium containing 0.25 M sucrose, 1 mM MgSO₄, 25 mM KCl, 1 mM ATP, 40 mM Tris-Mes of desired pH, and 5 μg (oats) or 25 μg protein (cauliflower) in a total volume of 0.5 ml. The assay was run for 30 min at 30°C.

2.4. Glucan synthetase II

Glucan synthetase II was assayed as in [11] with minor modifications. A total volume of 0.16 ml contained 0.15 M cellobiose, 0.10 M sucrose, 0.2 mM [³H]UDP-glucose (10⁴ Bq), 40 mM Tris-Mops of desired pH, and 20 μg protein. The assay was run for 25 min at 25°C.

2.5. Protein

Protein was measured as in [12] with 0.01% Triton X-100 present to solubilize membrane proteins; and with bovine serum albumin as standard.

3. RESULTS

3.1. pH optima of ATPase and glucan synthetase II

To exclude the possibility that a measured increase in enzyme activity on addition of detergent was merely due to a shift in pH optimum, it was necessary to check the pH dependency of the activities.

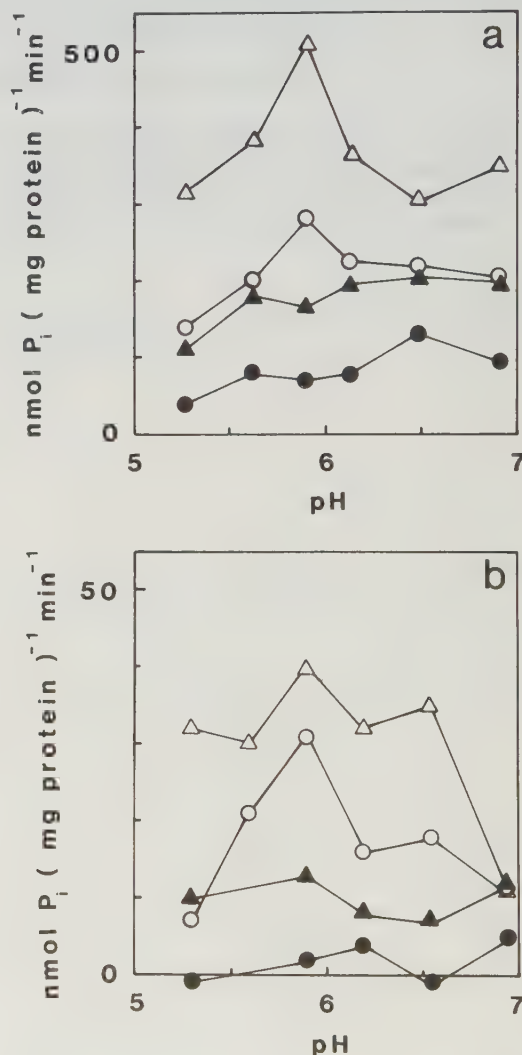


Fig.1. pH dependency of the K⁺-stimulated Mg²⁺-dependent ATPase activity of isolated plasma membrane vesicles from: (a) oat roots and (b) cauliflower inflorescences. The activity was assayed with either Mg²⁺ only (○, ●) or Mg²⁺ + K⁺ (△, ▲). (○, △) 0.01% Triton X-100 present.

The K^+ -stimulated Mg^{2+} -dependent ATPase is usually assayed at pH 6.0 or 6.5 [7,10]. We found the pH optimum to be around 5.9 for both plant materials in the presence of 0.01% Triton X-100 (fig.1a,b). This was the case both when the ATPase was assayed with Mg^{2+} only, and when assayed with Mg^{2+} and K^+ . In the absence of detergent the activities were low and no pH optimum was readily found. Thus, the addition of detergent stimulated ATPase activity in the whole pH interval (5.3–6.9), and the stimulation of activity at any fixed pH was not due to a shift in pH optimum.

For glucan synthetase II of cauliflower a pH optimum of 7.0 was found both with and without 0.01% Triton X-100 (fig.2b); and the activity was stimulated by detergent in the whole pH interval (6–8). With oat root, however, glucan synthetase II activity was inhibited by 0.01% Triton X-100; and the pH optimum was shifted from 7.5 to 7 (fig.2a).

3.2. Enzyme latency

A broad range of Triton X-100 concentrations was used, since the detergent could be expected to stimulate enzyme activity at low concentrations and inhibit at higher concentrations.

Mg^{2+} -dependent ATPase activity, as well as the K^+ -stimulated Mg^{2+} -ATPase activity, was stimulated by increasing concentrations of Triton X-100 up to 0.05%; at higher concentrations an inhibitory effect became evident (fig.3a,b). The latency curves obtained with cauliflower and oat root were similar, but the activities obtained with oat root material were higher, particularly the rates without detergent. Assuming that the ATP-binding site of the ATPase is at the cytoplasmic side of the plasma membrane, the latencies observed at 0.05% Triton X-100 suggest that the plasma membrane preparation obtained from cauliflower contained about 90% sealed right-side-out vesicles, while that from oat root contained about 70% sealed right-side-out vesicles, sealed in this case being defined as not leaky to ATP.

It was recently shown [13] that relatively high concentrations (0.5 mM) of vanadate may be used to inhibit completely plasma membrane-bound K^+ -stimulated Mg^{2+} -ATPase in oat root microsomes, without affecting the activities of other K^+ - or Cl^- -stimulated Mg^{2+} -ATPases. Under these conditions, the K^+ -stimulated

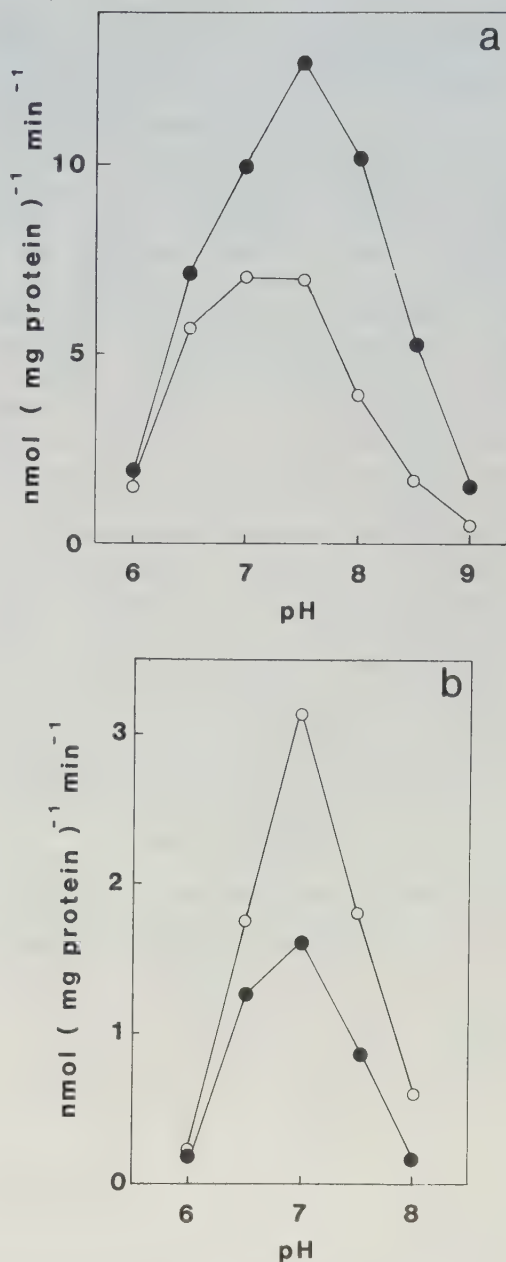


Fig.2. pH dependency of glucan synthetase II activity of isolated plasma membrane vesicles from: (a) oat roots and (b) cauliflower inflorescences. (○) 0.01% Triton X-100 present.

Mg^{2+} -ATPase activity present in oat root plasma membrane vesicles purified by phase partition was completely inhibited (M. Sommarin, personal communication); and the activity in cauliflower

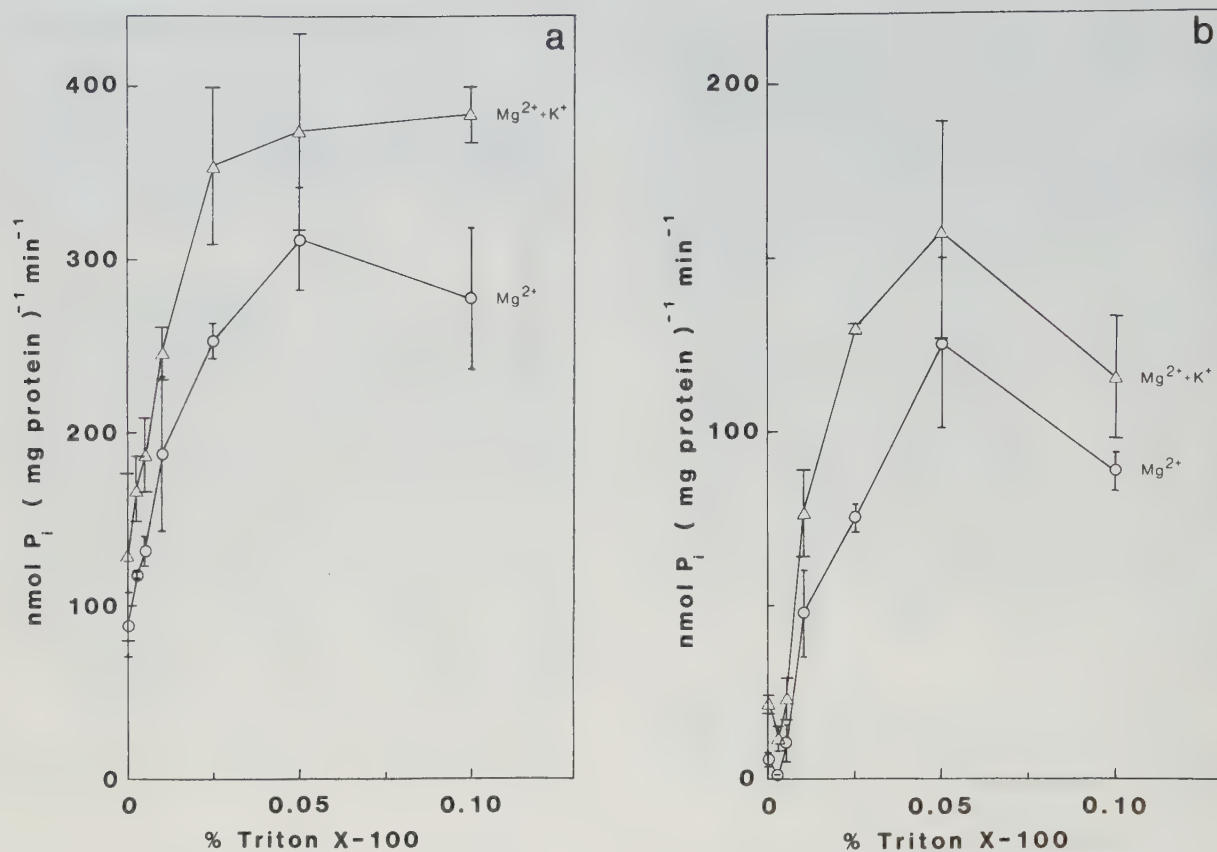


Fig.3. Latency of K⁺-stimulated Mg²⁺-dependent ATPase activity of isolated plasma membrane vesicles from: (a) oat roots and (b) cauliflower inflorescences. The activity was assayed with either Mg²⁺ only (○), or Mg²⁺ + K⁺ (Δ). Each value represents the mean and maximum variation of two independent experiments.

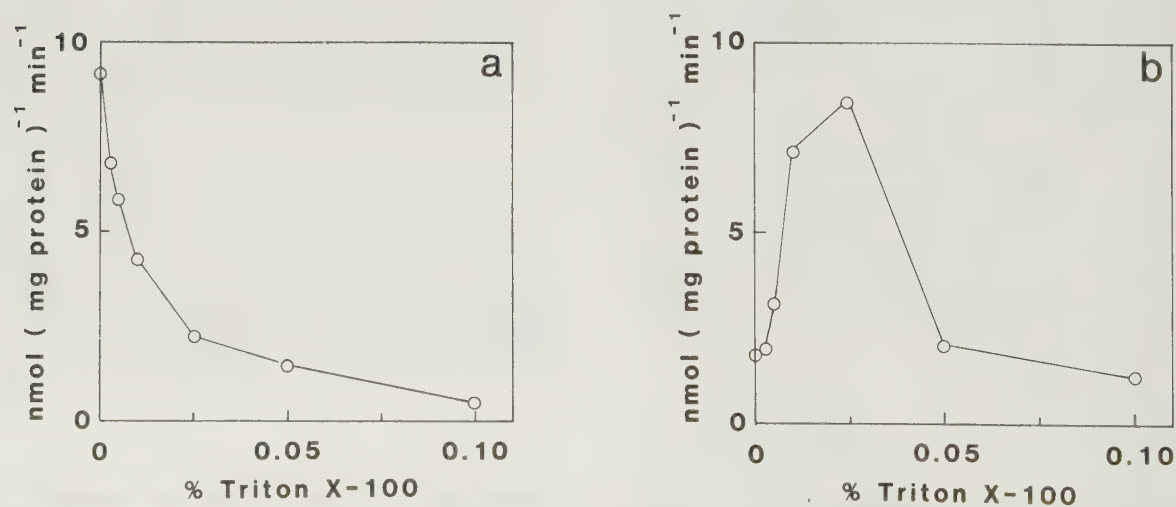


Fig.4. Latency of glucan synthetase II activity of isolated plasma membrane vesicles from: (a) oat roots and (b) cauliflower inflorescences.

plasma membrane was inhibited to more than 90%, whereas azide (1 mM), a known inhibitor of mitochondrial ATPase, had no effect on the activities. Moreover, ammonium molybdate (0.1 mM), an inhibitor of non-specific acid phosphatases, had no effect on the activity in oat plasma membrane (M. Sommarin, personal communication). Thus, there are strong indications that the measured ATPase activities are associated with the plasma membrane, and not due to enclosure of mitochondrial or tonoplast membranes, or of soluble ATPase within plasma membrane vesicles.

Glucan synthetase II of oat root was strongly inhibited by Triton X-100 (fig.4a); and not even at the lowest concentration (0.0025%) of detergent was any latency observed. With cauliflower, however, the glucan synthetase II activity was stimulated several-fold up to 0.025% Triton X-100 (fig.4b); only at higher concentrations was inhibition observed. These results suggest that the UDP-glucose-binding site of glucan synthetase II is located on the same side of the plasma membrane as the ATP-binding site of the ATPase, i.e., most probably on the cytoplasmic side. In fact, the latency curves for glucan synthetase II and ($K^+ + Mg^{2+}$)-ATPase of cauliflower are almost identical below 0.025% Triton X-100, where inhibition of the former enzyme probably starts to become severe.

4. DISCUSSION

Results from recent studies on the sidedness of plasma membrane vesicles purified by density gradient centrifugation are conflicting. Authors in [14], using plasma membrane vesicles prepared from carrot protoplasts, obtained evidence from latency of concanavalin A binding, that their preparations contained a mixture of right-side-out and inside-out vesicles. Authors in [15], on the other hand, using the same assay for sidedness, found only right-side-out vesicles in their preparations, and suggested that all plasma membrane vesicles formed after homogenization of corn coleoptiles are right-side-out. This is questioned by the author in [16], using the same material and an assay based on *N*-1-naphthylphthalamic acid (NPA) binding. He provides evidence for a partial

separation of right-side-out and inside-out plasma membrane vesicles by polyethylene glycol-facilitated sedimentation. The presence of plasma membrane vesicles with different orientation in preparations obtained by density gradient centrifugation would not be unexpected, since this method separates particles according to density and not according to surface properties, as does phase partitioning [3].

About 50% of the plasma membrane vesicles prepared by density gradient centrifugation were not tightly sealed, as measured by latency of ATPase [14], and UDPase [15] activity. This may be due to the low and changing osmotic potential experienced by the vesicles in the gradient. By contrast, the use of phase partitioning allows the preparation of plasma membrane vesicles in an iso-osmotic environment throughout the procedure.

Thus, preparations with a large proportion of sealed vesicles, which are homogeneous with respect to sidedness, may be obtained by phase partitioning. With the present procedure right-side-out vesicles are obtained. However, if inside-out vesicles of plasma membrane are present in the homogenates, it should also be possible to purify these by a modification of the procedure.

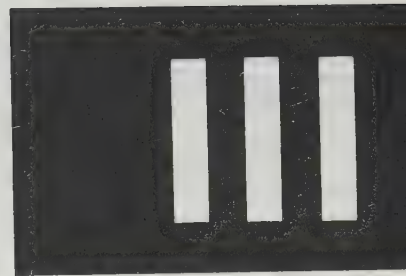
ACKNOWLEDGEMENTS

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Surface Properties of Right Side-Out Plasma Membrane Vesicles Isolated from Barley Roots and Leaves¹

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ABSTRACT

Highly purified plasma membrane vesicles were obtained from roots and leaves of 7-day-old light-grown barley (*Hordeum vulgare* L. cv Kristina) seedlings by partitioning of crude microsomal fractions in a dextran-polyethylene glycol two-phase system. Sodium dodecylsulfate polyacrylamide gel electrophoresis showed the polypeptide composition of plasma membranes from the two organs to be qualitatively similar, but with different relative amounts of some of the polypeptides. Between 80 and 100% of the K^+ , Mg^{2+} -ATPase activity was latent indicating that the vesicles were sealed and right side-out. The isoelectric points of the outer surface of root and leaf plasma membranes as determined by cross-partitioning were similar and quite acidic—about pH 3.6. In contrast, the net negative surface charge density at pH 7.0 as measured by 9-aminoacridine fluorescence differed significantly, being $-29 \text{ mC} \cdot \text{m}^{-2}$ for the leaf plasma membrane and only $-19 \text{ mC} \cdot \text{m}^{-2}$ for the root plasma membrane. As isolated, both types of plasma membrane vesicles had Ca^{2+} and Mg^{2+} bound to the outer surface as shown by the combined use of chelators and 9-aminoacridine fluorescence; however, the leaf plasma membrane had a relatively higher proportion of Ca^{2+} bound (0.57) than did the root plasma membrane (0.45). This difference probably reflects differences in the *in vivo* conditions as no chelator was present during the isolation procedure. Also Ni^{2+} could bind to the root vesicles as indicated by the effect of Ni^{2+} on 9-aminoacridine fluorescence, and by the binding of $^{63}\text{Ni}^{2+}$ (44 nanomoles bound per milligram protein) at 100 micromolar NiCl_2 .

Biological membranes carry a net negative surface charge at neutral pH. The isoelectric point of plant (15) and mammalian (13) mitochondrial membranes is at pH 4.7 to 5.4, and of thylakoid membranes at 4.1 to 4.7 (1, 35). The negative surface charge gives rise to a negative surface potential, the size of which depends on the net number of negative charges per unit surface area (the net surface charge density, measured in $\text{mC} \cdot \text{m}^{-2}$) as well as on the concentration and type of cations present in the solution with which the membrane surface is in contact. The presence of this surface potential affects the apparent K_m and the apparent pH optimum of membrane-bound enzymes (12, 38) and sometimes even the V_{max} (24). Other processes like protein

binding (31), insertion of newly synthesized proteins into membranes (34), and host-pathogen interactions (10) may also be influenced by the surface potential, *i.e.* by electrostatic interactions between the charged membrane surface and charged proteins. Thus, for studies of membrane structure and function it is clearly important to have information on the electrostatic properties of the membrane.

The electrostatic properties of thylakoid membranes have been studied extensively by Barber (2, and references therein). Electrostatic interactions at the outer surface of the inner membrane of plant mitochondria have also been described, particularly the specific and unspecific effects of cations on NADH oxidation (24, 28, and references therein). Although some work has appeared on the electrostatic properties of microsomes (*e.g.* 32) only a few recent reports have dealt with purified plasma membranes (15, 25, 26). This is particularly unfortunate as the plasma membrane forms the outer border of plant cells which is in contact with the variable external medium. In the roots, the plasma membrane is responsible for the discrimination between and the uptake of ions from the soil solution, and one might therefore expect the plasma membrane from roots to show special electrostatic properties when compared with plasma membranes from, *e.g.* leaves. The outer surface of the plasma membrane is also the first site of interaction between invading pathogens and the plant cell.

The plasma membrane preparations isolated by phase partitioning from various plant species and organs are highly purified (36; for review see Larsson [20]) and appear to consist mainly of sealed right side-out vesicles (21). The isolation of purified plasma membrane vesicles from green leaves and the establishment of their polypeptide composition (18) provides the possibility to compare plasma membranes from green and nongreen parts of the same plant. This makes it possible to ascertain how the differentiation into root and shoot affects properties of the plasma membrane.

In the present study, we have isolated plasma membrane vesicles from both roots and leaves of light-grown barley and compared their polypeptide composition, and isoelectric point, as well as other electrostatic properties.

MATERIALS AND METHODS

Chemicals. Dextran T500 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and PEG 3350 (earlier designated 4000) from Union Carbide. The anionic polymer sodium PEG-sulfonate and the cationic trimethylamino-PEG bromide were obtained from Aqueous Affinity, Lund, Sweden. $^{63}\text{NiCl}_2$ was obtained from Amersham Radiochemical Centre, England. 9-Aminoacridine was from Sigma (No. A-1135). All other chemi-

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cals were of analytical grade.

Preparation of Plasma Membrane Vesicles. Barley (*Hordeum vulgare* L. cv Kristina) was grown at 19°C in a low salt medium according to Wignarajah *et al.* (37), except for the use of light (70 w·m⁻² during a 12-h d). After 7 d, roots and leaves were harvested and plasma membrane vesicles prepared by differential centrifugation and partitioning in an aqueous dextran-PEG two-phase system as described by Kjellbom and Larsson (18). The composition of the phase system: 6.2% (w/w) Dextran T500, 6.2% (w/w) PEG 3350, 0.33 M sucrose, 3 mM KCl, 5 mM K-phosphate (pH 7.8) was the same for the purification of plasma membranes of both roots and leaves. All steps in the preparation procedure were carried out at 4°C. (For a detailed treatise of the method see Larsson [20]).

The purified plasma membrane vesicles were resuspended in 0.33 M sucrose and kept on ice or frozen in liquid N₂. Freshly prepared vesicles were used for most measurements. Exceptions were (a) SDS-PAGE and protein determinations (both frozen and unfrozen material have been used, without detectable differences), and (b) binding studies with ⁶³Ni²⁺ and IDPase assays (only frozen material in both cases). All measurements where the sidedness of the vesicles was of importance were performed in iso-osmotic media to avoid rupturing of the vesicles. Average yields of plasma membranes were 4.4 and 2.8 mg of protein from 125 g of roots and leaves, respectively.

SDS-PAGE was run on gradient gels (total amount of monomers, 12 to 20%; degree of cross-linking, 2.7%; gel dimensions 140·80·2.7 mm) in the buffer system of Laemmli (19) using a circulating running buffer at 12°C. During the passage through the stacking gel, a current of 5 mamp/gel was applied, and during the rest of the electrophoresis 7.5 mamp/gel. Total electrophoresis time was 20 h. Silver staining was essentially as described by Guevara *et al.* (16). Staining with Coomassie brilliant blue R-250 was according to standard procedures.

ATPase Activity. K⁺-Stimulated Mg²⁺-dependent ATPase activity was determined essentially as described by Hodges and Leonard (17). The assay medium was iso-osmotic and contained 0.25 M sucrose, 1 mM ATP, 40 mM Tris-Mes (pH 5.9), and 5 µg protein in a total volume of 0.5 ml, as well as 1 mM MgSO₄ and 25 mM KCl where indicated. The assay was run at 30°C for 30 min. Enzyme latency on addition of Triton X-100 was calculated according to Larsson *et al.* (21) (Fig. 3).

IDPase Activity. Latent IDPase was assayed at pH 7.5 according to Green (14), except that latency was tested by addition of detergent (0.05% [w/v] Triton X-100) rather than by letting the samples stand for 3 d at 4°C.

Determination of the Isoelectric Point. The isoelectric point of the plasma membrane vesicles was determined by cross-partitioning (1, 35). The vesicles were partitioned in two series of phase systems where the pH was varied using a citrate buffer. A relatively large electrostatic potential difference between the bulk phases was produced by including either a cationic or an anionic PEG derivative in the phase systems: In one series of phase systems trimethylamino-PEG bromide was used, in the other sodium PEG-sulfonate.

A final two-phase system weighed 2.00 g and contained: 6.8% (w/w) Dextran T500, 4.1% (w/w) PEG 3350, 2.7% (w/w) trimethylamino-PEG or PEG-sulfonate, 0.33 M sucrose, 2 mM citrate buffer at different pH, and sample. Fresh solutions of the PEG derivatives were always used to avoid any problems due to possible instability of these polymers in solution.

Fluorescence of 9-Aminoacridine. The fluorescence of 9-aminoacridine was measured as described by Møller *et al.* (26) in a medium containing 0.3 M sucrose, 5 mM MOPS²-KOH (pH 7.0),

20 µM 9-aminoacridine and plasma membrane vesicles from roots or leaves as indicated. The relative effect of chelators, EDTA and EGTA, as well as the effect of titrations with cations, especially Ni²⁺, was measured as specified in the legends to the figures and tables. The surface charge density at neutral pH was determined as described by Møller *et al.* (26) and the calculations performed as in Chow and Barber (9).

Binding of ⁶³Ni²⁺. Binding of Ni²⁺ to root plasma membrane vesicles was also determined using the radioactive tracer ⁶³Ni²⁺. The binding solution contained 0.3 M sucrose, 5 mM MOPS-KOH (pH 7.0), and ⁶³Ni-labeled NiCl₂ (68.4 GBq/mol) in the concentration range 0 to 100 µM. Incubation of vesicles in 0.5 ml binding solution was stopped by addition of 3 ml ice-cold desorption solution (0.3 M sucrose, 5 mM MOPS-KOH [pH 7.0], and 100 µM unlabeled NiCl₂) after 10 min at room temperature. The vesicles were immediately collected on a 0.2 µm Millipore filter (EGWP 025). For each Ni²⁺ concentration, a filter background was run without sample. The filters were dried and the radioactivity measured by liquid scintillation counting.

Protein Determination. Protein was determined with a modified Bearden (3) procedure, using Triton X-100 to solubilize the membrane proteins. The fractions were first incubated with 0.1% (w/v) Triton, and then water and reagents were added to bring the Triton concentration to 0.01%. This final concentration of Triton X-100 did not disturb the assay. BSA was used as the standard.

RESULTS

Polypeptide Composition. The crude microsomal fraction from barley leaves contained several dominant polypeptides which were completely absent in the final plasma membrane fraction (Figs. 1 and 2) in agreement with earlier results (18). By comparison with the polypeptide pattern of chloroplast thylakoids isolated from barley leaves (Fig. 1) the more prominent bands in the microsomal fraction may be identified as thylakoid polypeptides. The absence of these major thylakoid polypeptides in the plasma membrane fraction underlines the efficiency of the phase partitioning procedure in separating thylakoid fragments from plasma membranes. Similarly, several bands were more pronounced in the plasma membrane fraction from roots as compared with the root microsomal fraction (Fig. 2), whereas other bands were only found in the microsomal fraction.

The polypeptide composition of the plasma membranes from roots and leaves appears to be nearly identical (Fig. 2; compare tracks 2 and 3) although the relative abundance of some of the polypeptides vary. Thus, the bands at 16, 27, 44, and 85 kD are stronger in the leaf plasma membrane, whereas the bands at 22, 37, and 110 kD are stronger in the root plasma membrane (indicated with arrowheads in Fig. 2). Prominent polypeptides in both root and leaf plasma membranes are found at 26, 28, 31, 40, 56, and 73 kD. Note the difference in staining obtained with silver and Coomassie brilliant blue (compare Figs. 1 and 2). For instance, the 26, 27, and 28 kD polypeptides of the leaf plasma membrane appear as major bands with silver staining (Fig. 2) but are barely detected with Coomassie brilliant blue (Fig. 1).

IDPase Activity. Latent IDPase, a suggested marker for the Golgi apparatus (14, 27), could not be detected in the plasma membrane fractions nor in the microsomal fractions, possibly due to a low content of Golgi in the plant material used. Furthermore, most of the Golgi should have been removed already in the first centrifugation step (14, 27). Nonlatent IDPase, however, was detected in both root and leaf microsomal fractions (960 and 250 nmol (mg protein)⁻¹·min⁻¹, respectively) as well as in the corresponding plasma membrane fractions (180 and 80 nmol (mg protein)⁻¹·min⁻¹, respectively). Nonlatent IDPase activity of the latter order has earlier been reported in plasma membrane-enriched fractions free from Golgi (27).

² Abbreviations: MOPS, 3-(N-morpholino)propanesulfonic acid; (DM)Br₂, decamethylene-1,10-bis(trimethylammonium) bromide.

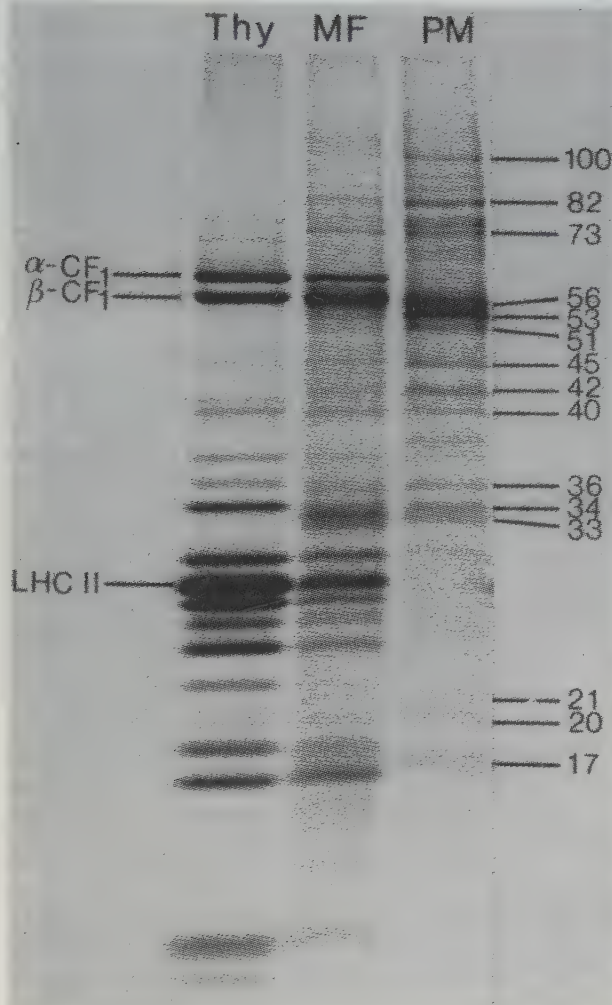


FIG. 1. SDS-PAGE of plasma membranes (PM) obtained from barley leaves. The polypeptide patterns of the microsomal fraction (MF) used as starting material, and chloroplast thylakoids (Thy) are shown for comparison. Mol wt (kD) of prominent plasma membrane polypeptides are indicated to the right. To the left, the positions of some major thylakoid polypeptides, namely the α - and β -subunits of the ATP synthase (α -CF₁, β -CF₁), and the apoprotein of the Chl a/b-protein complex of PSII (LHC II), are indicated. Note the absence of thylakoid polypeptides in the plasma membrane fraction. Tracks 2 and 3 were loaded with 100 μ g of protein from the plasma membrane and microsomal fractions, respectively. The gel was stained with Coomassie brilliant blue R-250.

Polarity of Vesicles. From previous studies we know that plasma membrane preparations consist of vesicles (e.g. 18, 36). It is important to determine the sidedness and degree of leakiness of such vesicles before characterizing their surface properties. We used the latency of the K^+ , Mg^{2+} -ATPase to do this (21). In an iso-osmotic medium, the K^+ , Mg^{2+} -ATPase activity was very low for both root and leaf plasma membrane preparations (Fig. 3). When Triton X-100 was added to rupture the vesicles the K^+ , Mg^{2+} -ATPase activity increased several-fold. This indicates that: (a) the active site of the K^+ , Mg^{2+} -ATPase is located on the inside of the vesicles and that these therefore are right side-out, since the active site of this ATPase is assumed to be located on

the cytoplasmic side of the plasma membrane; (b) the vesicles are tightly sealed so that no Mg -ATP can approach the active site of the enzyme. The degree of latency was found to be between 80 and 100% for the plasma membrane vesicles from both roots and leaves (Table I; Fig. 3).

The plasma membrane vesicles used in this work thus have the apoplastic side exposed to the medium and all of the following data pertain to this surface.

The Isoelectric Point of the Plasma Membrane. The isoelectric point of the outer surface of the vesicles was determined by cross-partitioning and found to be at about pH 3.6 for both the root and leaf plasma membrane (Fig. 4; Table I). At the isoelectric point, the partition of the vesicles between the upper and lower phase is independent of the electrostatic potential difference between the phases created by the charged polymers; i.e. the isoelectric point is identical to the cross-point of the two curves in Figure 4. At the cross-point, root plasma membrane vesicles were mainly in the upper phase whereas leaf plasma membrane vesicles were more evenly distributed (Fig. 4). This cross-point represents the net-charge-independent partitioning of the vesicles and the difference indicates that the outer surface of root and leaf plasma membranes differ in hydrophobic/hydrophilic properties (35).

The data obtained for the partitioning at the lowest pH values tested (around pH 3) repeatedly deviated from the curve given by the other values (Fig. 4). This deviation might be due to rearrangements in the membrane at this unphysiological pH, or alternatively be due to release of divalent cations bound by carboxyl groups (which are titrated at this pH), thus changing the net charge of the membrane surface.

The Surface Charge Density of the Plasma Membrane. The surface charge density was about 50% more negative for the outer surface of leaf than for root plasma membrane vesicles as determined by 9-aminoacridine fluorescence at pH 7.0 (Table I). Thus, although the type and relative amounts of charged groups are similar for root and leaf vesicles, as indicated by the very similar isoelectric point (Fig. 4; Table I), the number of charged groups per unit area differ.

Bound Divalent Cations. The fluorescence of 9-aminoacridine can be used to monitor changes in the surface potential of membranes as originally shown by Searle *et al.* (30). An increase in the size of the surface potential is followed by a decreased fluorescence and vice versa. Thus, when bound divalent cations are removed from the surface of membranes by chelators, this is observed as a decrease in fluorescence as the membranes become more negative (23, 25). Since EGTA is specific for Ca^{2+} , whereas EDTA removes both Ca^{2+} and Mg^{2+} , the two dominant divalent cations on the membrane surface, the relative effects of EGTA and EDTA gives information on the relative amounts of bound Ca^{2+} and Mg^{2+} .

Titration of barley plasma membrane vesicles with chelators in the presence of 9-aminoacridine showed that both root and leaf vesicles are isolated with bound Ca^{2+} and Mg^{2+} (Fig. 5; Table II). Since the vesicles were prepared in the absence of any chelator (even in the homogenization medium; the 5 mM Pi used in the phase system is a weak complexor and allows the presence of 100 μ M free Ca^{2+}), the amounts of bound divalent cations are probably similar to those found *in vivo*. It is therefore interesting that the relative effects of EGTA and EDTA are different for root and leaf vesicles. Plasma membrane vesicles from barley leaves appear to have relatively less Mg^{2+} bound to their outer surface than do plasma membrane vesicles from roots as suggested by the smaller relative additional effect of EDTA (Fig. 5; Table II).

The Binding of Added Cations. When cations are added to membranes suspended in a low-cation medium they cause a decrease in the size of the surface potential due to electrostatic

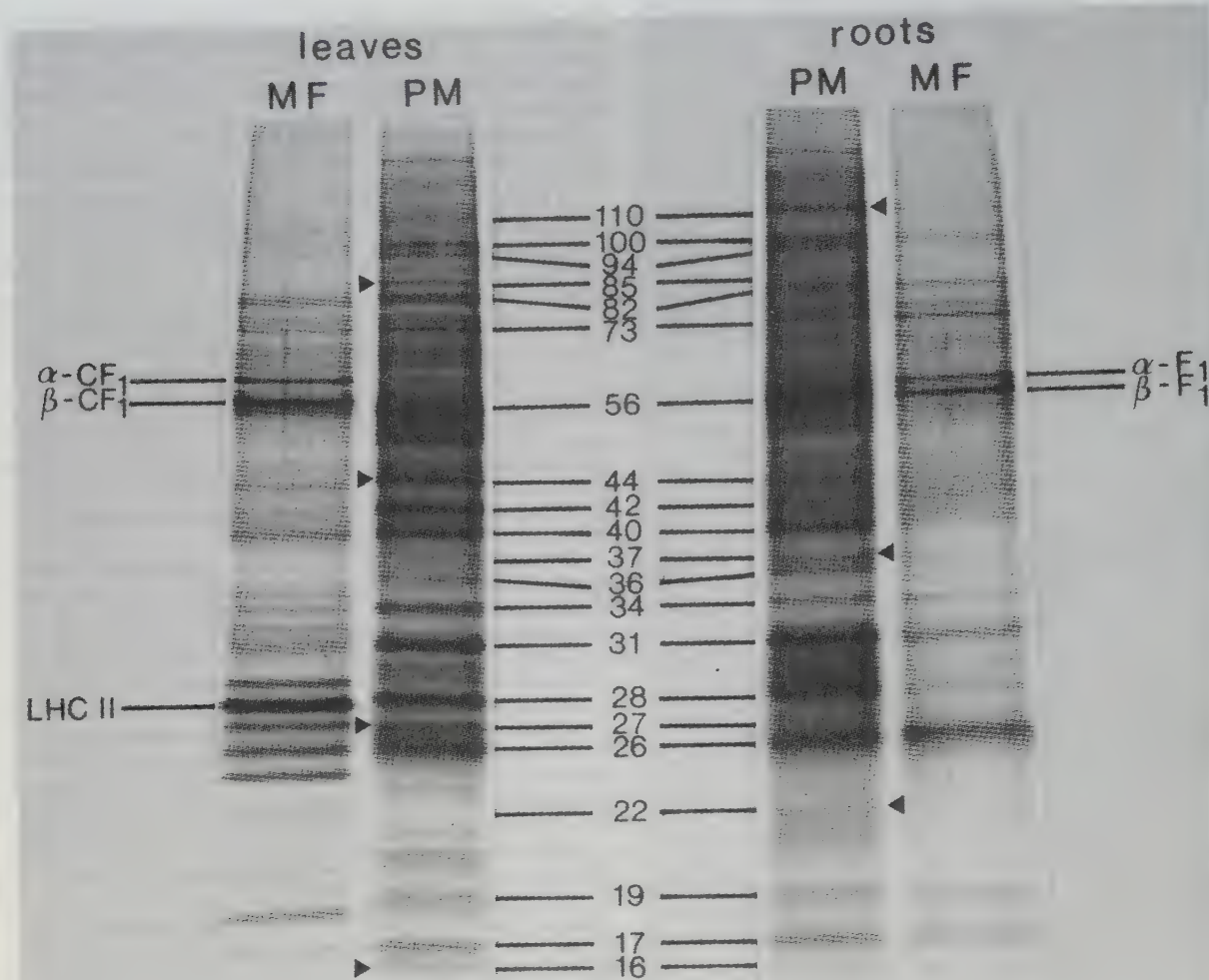


FIG. 2. SDS-PAGE of plasma membranes (PM) and microsomal fractions (MF) from barley leaves and roots. The mol wt (kD) of prominent plasma membrane polypeptides are indicated in the middle. Note that leaf and root plasma membranes were run on neighboring tracks; the photograph has been cut to facilitate the indication of mol wt. Major differences between leaf and root polypeptides are indicated by arrowheads. The 56 and 58 kD polypeptides of the root microsomal fraction are tentatively identified as the α - and β -subunits, respectively, of the mitochondrial ATP synthase (α -F₁, β -F₁). Other abbreviations as in Figure 1. Ten μ g protein of each fraction was loaded on the gel, which was silver stained (16).

screening. This can be observed as an increase in 9-aminoacridine fluorescence. In the presence of 100 μ M EDTA to remove all bound divalent cations (Fig. 5), the addition of 0 to 20 mM KCl or 0 to 600 μ M (DM)Br₂ gave hyperbolic increases in 9-aminoacridine fluorescence (Fig. 6) consistent with the low binding constants for K⁺ and (DM)²⁺. The addition of low amounts (0–50 μ M) of CaCl₂ or NiCl₂, on the other hand, had very little effect probably due to complexing with residual EDTA in the solution

(23). Above 50 μ M, the effect of Ca²⁺ and Ni²⁺ was hyperbolic and both ions were much more efficient at affecting the surface potential than (DM)²⁺ (at least 4-fold, [Fig. 6]) probably due to binding of Ni²⁺ and Ca²⁺ to the membrane surface. The concentration of Ca²⁺ and Ni²⁺ at which the half maximal effect was observed was 90 ± 14 (mean $\pm$ SD, $n = 4$) and 73 ± 13 ($n = 5$) μ M, respectively.

Ni²⁺ was chosen as a representative of the heavy metal ions

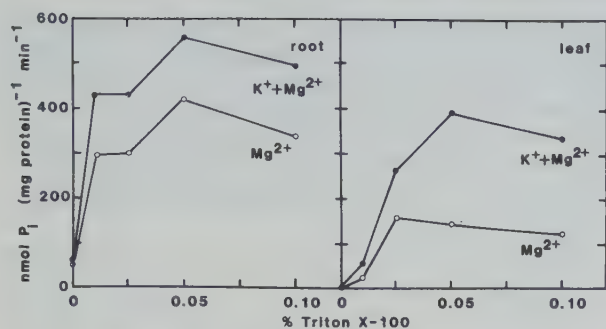


FIG. 3. Latency of the K^+ , Mg^{2+} -ATPase activity in plasma membrane vesicles from barley roots (left) and leaves (right). The vesicles were assayed in an iso-osmotic medium with addition of Triton X-100 as indicated. Both the activities in the presence of Mg^{2+} only ($\circ$), and in the presence of $Mg^{2+} + K^+$ ($\bullet$) were recorded. The latency was calculated as:

$$\frac{(\text{Activity with Triton}) - (\text{activity without Triton})}{(\text{Activity with Triton})} \cdot 100\%$$

see Table I.

Table I. Properties of Plasma Membrane Vesicles from Barley Roots and Leaves

Values are given as mean $\pm$ SD (number of independent preparations).

Parameter	Root	Leaf
Latency of K^+ , Mg^{2+} -ATPase ^a	87 $\pm$ 3 (2)	100 $\pm$ 0 (2)
Isoelectric point ^b	3.55 $\pm$ 0.10 (4)	3.65 $\pm$ 0.10 (2)
Partition at the cross-point (%) ^b	76 $\pm$ 20 (4)	41 $\pm$ 11 (2)
Surface charge density (mC $\cdot$ m ⁻²) ^c	-19 $\pm$ 1 (4)	-29 $\pm$ 3 (3)

^a See Figure 3. ^b See Figure 4. ^c Determined according to Chow and Barber (9) as described by Møller *et al.* (26).

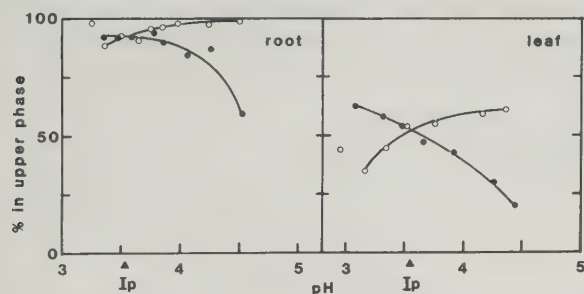


FIG. 4. Determination of the isoelectric point (Ip) of the outer surface of plasma membrane vesicles from barley roots (left) and leaves (right) by cross-partitioning. Right side-out plasma membrane vesicles were partitioned in phase systems of different pH containing either trimethylamino-PEG (positive upper phase [$\circ$]) or PEG-sulfonate (negative upper phase [$\bullet$]). At the isoelectric point of the membrane surface the partition is independent of the electrostatic potential difference between the phases created by the charged polymers *i.e.* the isoelectric point is identical to the cross-point. The unexpected increase in partition at very low pH (unconnected points) is treated in "Discussion".

which contaminate our environment to an increasing extent and whose uptake into plants might cause structural and functional changes. The binding of Ni^{2+} to the outer surface of the plasma membrane of the root is clearly an important step in this uptake process and we therefore attempted to determine at what concentration binding of Ni^{2+} to the plasma membrane occurred.

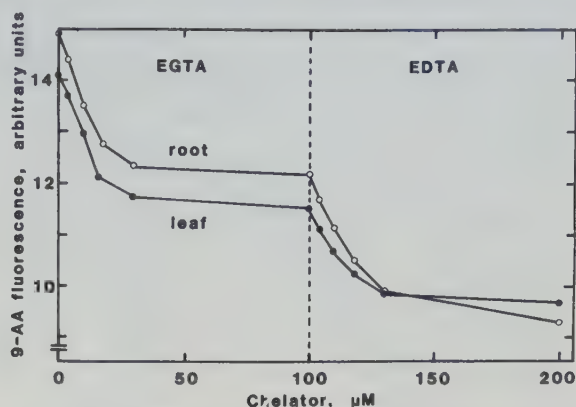


FIG. 5. Monitoring bound divalent cations on the outer surface of plasma membrane vesicles from barley roots ($\circ$, 62 μ g protein/ml) and leaves ($\bullet$, 77 μ g protein/ml). Samples were suspended in an iso-osmotic medium containing 9-aminoacridine (see "Materials and Methods"), chelators were added to the final concentration indicated, and the changes in 9-aminoacridine fluorescence recorded.

Table II. Monitoring of Bound Divalent Cations on the Outer Surface of Plasma Membrane Vesicles from Barley Roots and Leaves

Experimental details as in Figure 5.

Parameter	Root	Leaf
Number of preparations	3	4
Protein concentration in assay (μ g/ml)	62-114	77-130
Effect of EGTA	2.5 $\pm$ 0.4 ^a	2.5 $\pm$ 0.4
Additional effect of EDTA	3.1 $\pm$ 0.2	2.0 $\pm$ 0.5
EGTA/EDTA ratio	0.45 $\pm$ 0.03	0.57 $\pm$ 0.10

^a Arbitrary units, mean $\pm$ SD.

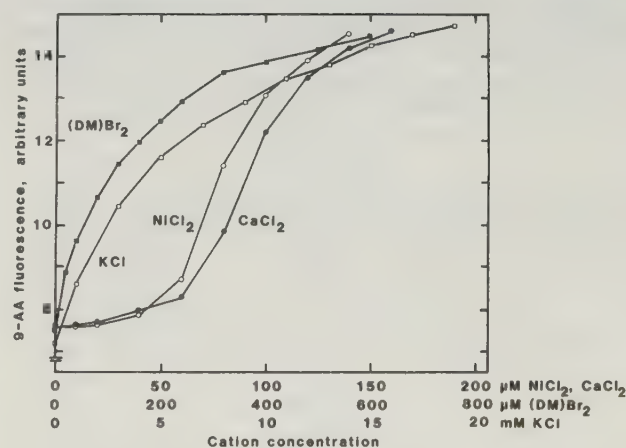


FIG. 6. Effect of cations on the surface potential of the outer surface of plasma membrane vesicles from barley roots. The vesicles (92 μ g protein/ml) were suspended in the medium described in "Materials and Methods" plus 100 μ M EDTA, salts were added to the final concentrations indicated, and the changes in 9-aminoacridine fluorescence recorded.

These experiments were performed in the absence of EDTA to leave the membranes with their natural complement of bound divalent cations (see above) and to avoid interference from EDTA (as observed in Fig. 6).

Two methods were used to follow the binding of Ni^{2+} : (a) titration of 9-aminoacridine fluorescence, and (b) binding of the

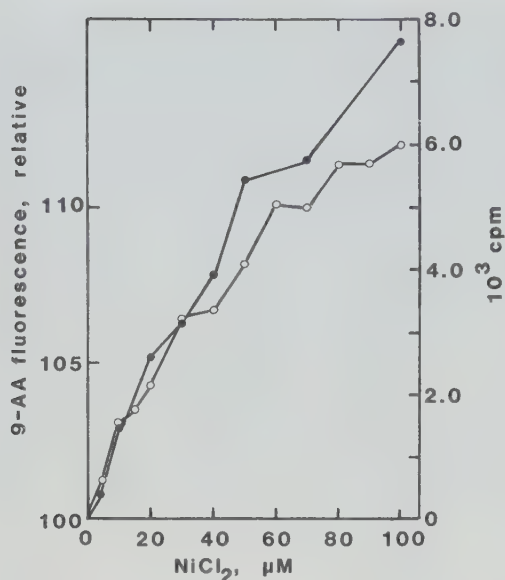


FIG. 7. Binding of Ni^{2+} to the outer surface of plasma membrane vesicles from barley roots. Binding was monitored using 9-aminoacridine fluorescence or $^{63}\text{NiCl}_2$ as described in "Materials and Methods". The curve showing the change in 9-aminoacridine fluorescence (O) is the average of four individual experiments (identical titrations with four separate samples containing 170 μg protein/ml). These are normalized at zero NiCl_2 to cancel out small differences in the initial fluorescence and the values given are relative. Each point for the $^{63}\text{NiCl}_2$ curve (●) is the average of two separate samples (containing 149 μg protein/ml). Similar curves were observed with other plasma membrane preparations.

radioactive $^{63}\text{Ni}^{2+}$. The effect of Ni^{2+} on 9-aminoacridine fluorescence (Fig. 7) never gave rise to a smooth hyperbolic curve, however, as observed with K^+ and $(\text{DM})^{2+}$ (compare Figs. 6 and 7). The experiments with $^{63}\text{Ni}^{2+}$ confirmed that binding took place (order of magnitude of 44 nmol Ni^{2+} /mg protein at 100 μM NiCl_2) and again the curves were not smooth (Fig. 7).

DISCUSSION

Plasma membranes prepared by partitioning in aqueous two-phase systems are in general very pure, and have been shown to be practically free from contamination by intracellular membranes (for review see Larsson [20]). Thus, even Chl-free preparations of plasma membrane are readily obtained from light-grown barley leaves (18; Fig. 1), and the close similarity in the polypeptide composition between root and leaf plasma membranes (Fig. 2) indicates that also the root plasma membrane preparation was very pure. Although the polypeptide composition appears to be qualitatively the same for root and leaf plasma membranes, the relative amounts of some of the polypeptides differed (Fig. 2). However, the overall similarity in polypeptide composition indicates that the function of the plasma membrane is similar in roots and leaves and that the two membranes differ mainly in the balance between the different processes taking place at their surfaces. The extensive similarity between plasma membranes from different organs of the same plant is consistent with recent observations that differentiation in general only causes the appearance/disappearance of relatively few polypeptides (11).

Plasma membranes from both roots and green leaves show bands in the 100 kD region where a subunit of the $\text{K}^+, \text{Mg}^{2+}$ -ATPase is found (33). However, none of these polypeptides belong to the main bands which are all found at lower mol wt in

both root and leaf plasma membranes. It is not surprising that the ATPase constitutes only a small percentage of the plasma membrane protein since an ATPase may well have a high turnover number, making each molecule highly efficient. Assuming that the barley ATPase has the same specific activity as that of the *Neurospora* plasma membrane (140 μmol [mg protein] $^{-1}$ min $^{-1}$, calculated from the purification scheme of Bowman *et al.* [7]) the barley ATPase should constitute less than 0.5% of the plasma membrane protein.

Almost all of the $\text{K}^+, \text{Mg}^{2+}$ -ATPase activity was latent in both types of vesicles (Fig. 3; Table I). This is consistent with results on plasma membrane preparations from cauliflower influences and oat roots (21), and shows that the vesicles are sealed (the substrate cannot penetrate to the active site of the ATPase) and right side-out. The latter conclusion is based on the assumption that the ATPase is located on the inner, cytoplasmic side of the plasma membrane. Unfortunately, the active site of the other established plasma membrane marker enzyme, glucan synthetase II (EC 2.4.1.34), also appears to be located on the cytoplasmic side (21). Thus, there is presently no known enzyme marker activity for the outer surface of the plasma membrane.

The isoelectric point for both root and leaf plasma membrane vesicles was at about pH 3.6 (Fig. 4; Table I) which is very close to the value of pH 3.4 obtained for the outer surface of the spinach leaf plasma membrane (35) also using cross-partitioning, but significantly lower than the pH 4.4 observed on plasma membranes from pea by Griffing and Quatrano (15) using isoelectric focusing. This latter technique may not, however, give the correct isoelectric point (29). It should be noted that the isoelectric point only gives information on the net surface charge at that particular pH (where it is zero per definition) and not at other pH values, in contrast to what has been implied by, *e.g.* Griffing and Quatrano (15). Therefore the net surface charge density of a membrane surface must be determined by a separate technique as done here using the fluorescence of 9-aminoacridine (Table I). The outer surface of plasma membrane vesicles from barley leaves was 50% more negative at pH 7.0 than that of root plasma membrane vesicles in spite of their identical isoelectric point (Table I; Fig. 4) and similar polypeptide pattern (Fig. 2). Root and leaf plasma membrane vesicles also differed markedly in their partitioning at the isoelectric point (Fig. 4; Table I) indicating a difference in hydrophobic/hydrophilic properties (35). Since the polypeptide patterns were relatively similar, the large differences in surface net charge density and hydrophobic/hydrophilic properties may be due to differences in the lipid composition of the membranes, a property not investigated so far. The barley leaf plasma membrane is a very lipid-rich membrane (70% w/w, [18]) why the contribution to the surface properties from the lipid fraction should be important.

The values of net surface charge density for the outer surface of barley plasma membranes (Table I) are compared to other values from the literature (Table III). All values presented were estimated by use of 9-aminoacridine fluorescence (2, 4, 9, 22, 26) since it is difficult to compare absolute values determined using different techniques, *e.g.* particle electrophoresis consistently gives lower values for the surface charge density probably due to the presence of the shearing layer (2, 9). Barley root plasma membrane vesicles have a similar surface charge density as plasma membrane vesicles and mitochondria from wheat roots which appear to belong to the membranes of intermediate charge (Table III). In contrast, the surface charge density of barley leaf plasma membrane vesicles resembles that of oat root plasma membrane and Jerusalem artichoke mitochondria. The surface charge density of different regions of the thylakoid membrane has been reported to span the entire range found for plasma membranes and mitochondria (Table III). The inside surface of plasma membrane vesicles (*i.e.* the cytoplasmic surface) is more

Table III. Surface Charge Density of Plant Membranes

Data were all measured by the use of 9-aminoacridine fluorescence.

Membrane	Source	Surface	
		Outer	Inner
		$mC \cdot m^{-2}$	
Plasma membranes	Wheat roots ^a		
	Low K ⁺	-21	-32
	High K ⁺	-19	-24
	Oat roots ^a	-29	-38
	Barley roots ^b	-19	
Mitochondria	Barley leaves ^b	-29	
	Jerusalem artichoke tubers ^a	-33	
Thylakoid membranes	Wheat roots ^c	-19 to -21	
	Pea leaves ^d	-21 (randomized thylakoids)	-37 (appressed region)

^a Møller et al. (26).^b This paper.^c Bérczi et al. (4).^d Mansfield et al. (22).

negative than the outer surface (Table III).

Both types of vesicles investigated in the present study had divalent cations bound to their outer surface as revealed by the reduction in 9-aminoacridine fluorescence (= increase in the size of the surface potential) upon addition of chelators (Fig. 5). The difference in the EGTA/EDTA ratio between root and leaf vesicles (Fig. 5; Table II) indicates that leaf vesicles have a relatively lower proportion of bound Mg²⁺ on their outer surface. Wheat leaves contain at least twice as much Ca²⁺ as wheat roots (6) and since the concentration of free Ca²⁺ is thought to be in the micromolar range more than 99% of this total Ca²⁺ must be bound. This would point to the existence of relatively more Ca²⁺-binding sites in the leaves than in the roots of cereals consistent with our observation (Table II). These bound divalent cations would reduce the net surface charge density *in vivo* (the values in Table II were determined in the presence of enough EDTA to strip the vesicles' outer surface of divalent cations). The electrostatic interaction between cations and charged cell membranes could be important for the regulation of the ion content in plant cells (4, 5). Of course, specific effects of membrane-bound divalent cations are also important; one could here mention the external NADH dehydrogenase in plant mitochondria which is dependent on bound Ca²⁺ (23).

The binding of Ni²⁺ to the outer surface of the root plasma membrane would be the first step in the uptake of Ni²⁺ and this uptake has been reported to have two phases in the concentration range 0 to 100 μM (8). It was therefore of interest to see if the binding showed several phases. The effect of Ni²⁺ on 9-aminoacridine fluorescence was never smoothly hyperbolic as would be expected for pure electrostatic screening (see, e.g. Fig. 6, curves with KCl and [DM]Br₂) and it appeared as if several phases could indeed be observed in the absence of EDTA (Fig. 7) which interfered with the assay (Fig. 6). The use of ⁶³Ni²⁺ also did not give rise to smooth binding curves (Fig. 7) and we therefore think that there may be several classes of binding sites with different affinities for Ni²⁺ on the outer surface of plasma membrane vesicles from barley roots.

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IV

CYTOCHROME P-450/420 IN PLANT PLASMA MEMBRANES: A POSSIBLE COMPONENT OF THE BLUE-LIGHT-REDUCIBLE FLAVOPROTEIN-CYTOCHROME COMPLEX*

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Abstract—Carbon monoxide difference spectra and pyridine binding spectra indicate the presence of cytochrome P-450/420 in plasma membranes from cauliflower inflorescences. Mild lithium dodecylsulfate polyacrylamide gel electrophoresis shows only one heme staining band in the plasma membrane fraction at an apparent molecular weight of 93 kiloDalton. This band is suggested to be due to a cytochrome P-450/420 dimer, in view of the known molecular weights of animal cytochromes P-450/420. The plasma membrane-bound cytochrome P-450/420 is probably identical to the blue-light-reducible b-type cytochrome of plant plasma membranes, which has been inferred to have a role in photomorphogenesis.

INTRODUCTION

Many physiological responses in plants are regulated by blue light. However, the nature of the photoreceptor(s) and the way(s) the light signal is transduced into a physiological response is essentially unknown. One possible photoreceptor has been tentatively identified as a flavoprotein-cytochrome complex, and this is the best characterized candidate so far (for reviews, see Briggs and Iino, 1983; Widell, 1985). The cytochrome of this complex is reduced by blue light, supposedly *via* the flavoprotein moiety. This light-reducible b-type cytochrome has been localized to the plasma membrane in several higher plants (Leong *et al.*, 1981; Lundborg *et al.*, 1981; Widell and Larsson, 1981, 1983; Widell *et al.*, 1982, 1983; Kjellbom and Larsson, 1984), although blue-light-reducible cytochrome is also found in intracellular membranes (Widell *et al.*, 1983). Spectral analysis of the plasma membrane-bound cytochrome in cauliflower inflorescences (Widell *et al.*, 1983) showed that it had a difference spectrum [dithionite-(or blue light)-reduced-minus-oxidized; 77 K] similar to that of a cytochrome earlier identified as cytochrome P-420 in cauliflower microsomes (Rich and Bendall, 1975). Concomitantly, it was found that preparations of plant plasma membrane contained NAD(P)H-cytochrome *c* reductase activity (Lundborg *et al.*, 1981; Widell and Larsson, 1983) of an order which could not be ascribed to contamination by endoplasmic reticulum (Kjellbom and Larsson, 1984). Since the NADPH-cytochrome *c* reductase of the endoplasmic reticulum is a flavoprotein, the physiological substrate of which is cytochrome P-450 rather than cytochrome *c*, this prompted us to

look for the presence of cytochrome P-450/420 in the plant plasma membrane, and to investigate whether the plasma membrane-bound light-reducible cytochrome could be a cytochrome P-450/420.

MATERIALS AND METHODS

Preparation of membrane fractions from cauliflower inflorescences (*Brassica oleracea*, L.) was essentially as described earlier (Widell and Larsson, 1983). The cauliflower inflorescences were homogenized, and a mitochondrial pellet (1000–9000 g) and a microsomal pellet (9000–50 000 g) were obtained by differential centrifugation. The crude microsomal fraction was added to an aqueous polymer two-phase system [final composition: 6.5% (wt/wt) Dextran T500, 6.5% (wt/wt) polyethylene glycol 3350, 0.25 M sucrose, 2 mM KCl, 5 mM potassium phosphate, pH 7.8] and fractionated into plasma membranes and intracellular membranes. With the phase composition used, plasma membranes partitioned in the upper phase, whereas intracellular membranes partitioned at the interface and in the lower phase. To increase the purity of the fractions, the initial upper and lower phases were repartitioned twice with fresh lower and upper phase, respectively. The final upper and lower phases were diluted severalfold and the membranes collected by ultracentrifugation. The pellets were resuspended in appropriate media, and the fractions were used fresh or stored in liquid N₂ until use. The plasma membrane fraction obtained by phase partitioning is highly enriched in plasma membrane, and the intracellular membrane fraction depleted in plasma membrane (for a detailed treatise of the method, including contaminants in the plasma membrane fraction, see review by Larsson, 1985).

Carbon monoxide difference spectra were recorded with an Aminco DW 2 spectrophotometer operated in the split beam mode using dithionite to reduce the cytochromes, as described by Estabrook and Werrigloer (1978). The amounts of cytochrome P-450 and P-420 were calculated as described in Rich and Bendall (1975).

Pyridine binding spectra were obtained as described by Schenkman *et al.* (1967).

Lithium dodecylsulfate polyacrylamide gel electrophoresis was run on gradient gels (total amount of monomers, 12–20%; degree of cross-linking, 2.7%; gel dimension 180–140–1.5 mm) in the buffer system of

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Laemmli (1970) (except that lithium dodecylsulfate was substituted for sodium dodecylsulfate) using a circulating "running" buffer at 4°C. The solubilizing medium contained 2% (wt/vol) lithium dodecylsulfate, and 30 mM dithiothreitol instead of mercaptoethanol; the samples were solubilized at 4°C. During passage through the stacking gel, a current of 5 mA/gel was applied, and during the rest of the electrophoresis 7.5 mA/gel. Total electrophoresis time was 20 h. Heme staining with tetramethylbenzidine was according to Thomas *et al.* (1976). Protein staining with Coomassie brilliant blue R-250 was according to standard procedures.

Protein was determined with a modified Bearden (1978) procedure, using Triton X-100 to solubilize the membrane proteins. The fractions were first incubated with 0.1% (wt/vol) Triton, and then water and reagents were added to bring the Triton concentration to 0.01%. This final concentration of Triton X-100 did not disturb the assay. Bovine serum albumin was used as the standard.

Chemicals; Dextran T500 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and polyethylene glycol 3350 (earlier designated 4000) from Union Carbide, NY, U.S.A.

RESULTS

Carbon monoxide difference spectra of dithionite-reduced samples revealed large peaks at 421 nm in both the plasma membrane and intracellular membrane fraction, as well as in the microsomal fraction used as starting material for the former fractions (Fig. 1). In addition, a smaller peak appeared at 451 nm in

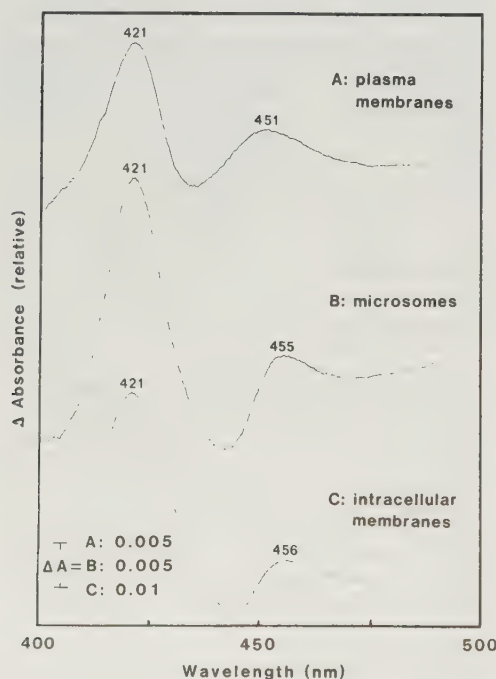


Figure 1. Carbon monoxide difference spectra of plasma membranes (2.5 mg protein/mL), crude microsomes (5 mg/mL) and intracellular membranes (5 mg/mL) from cauliflower inflorescences. The plasma membrane and intracellular membrane fractions were derived from the crude microsomal fraction by partition in an aqueous dextran-polyethylene glycol two-phase system.

the plasma membrane fraction and at 455–456 nm in the microsomal and intracellular membrane fractions. These spectra are consistent with the presence of cytochrome P-450 and its supposedly degraded form, cytochrome P-420, in all three membrane fractions. Similar spectra have been reported earlier with cauliflower microsomes and ascribed to endoplasmic reticulum-bound cytochrome P-450/420 by Rich and Bendall (1975), who found specific amounts of around 0.07 nmol/mg protein. The amounts of cytochrome P-450/420 in our preparations varied between 0.04 and 0.12 nmol/mg protein, but were relatively similar for plasma membranes, intracellular membranes and crude microsomes. The proportion of cytochrome P-450, however, was always somewhat higher in the plasma membrane fraction (around 20%) compared to the other two fractions (around 10% cytochrome P-450). That the 421 nm peak was due to the same molecular species as the 451 nm peak was demonstrated by addition of detergent (0.05% Triton X-100) to the samples which abolished most of the absorbance at 451 nm with a corresponding increase at 421 nm (not shown). This is in agreement with results on rat liver cytochrome P-450 (Omura and Sato, 1964), and similar results have earlier been reported for cauliflower microsomes (Rich and Bendall, 1975).

Further support for the presence of cytochrome P-450 in the plasma membranes is lent by the spectral changes obtained on addition of pyridine to oxidized samples. This procedure gave typical so called type II binding spectra (Fig. 2), with a minimum at 390 ± 4 and a maximum at 419 ± 1 for the plasma membranes ($n = 6$), at 389 ± 3 and 424 ± 2 , respectively, for the microsomal fraction ($n = 6$), and at 388 ± 2 and 424 ± 1 , respectively, for the intracellular membranes ($n = 2$). These binding spectra are very similar to those obtained with pyridine and rat liver cytochrome P-450 (minimum at 390 nm, maximum at 426 nm; Shenkman *et al.* 1967).

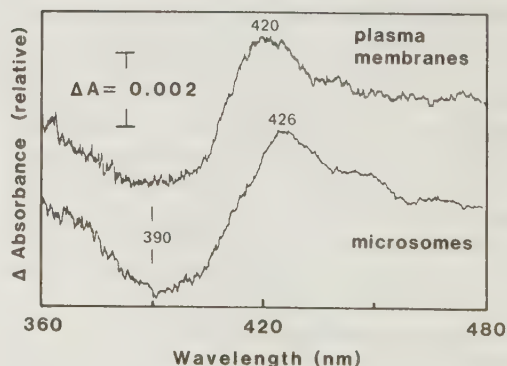


Figure 2. Spectral changes caused by the addition of pyridine (10 mM) to plasma membranes (1.6 mg protein/mL) and crude microsomes (2.6 mg/mL) from cauliflower inflorescences.

Rat liver cytochromes P-450 are known to have molecular weights varying between 44 and 57 kDa $\ddagger$ (Welton and Aust, 1974; Guengerich 1978). Mild solubilization of the membrane samples and lithium dodecylsulfate polyacrylamide gel electrophoresis at 4°C was used to determine the molecular weights of the heme staining polypeptides in the membrane fractions from cauliflower inflorescences. Mild solubilizing conditions were used in order to keep the heme groups bound to the polypeptides. Only one heme staining band was found in the plasma membrane fraction, with an apparent molecular weight of 93 kDa. In the mitochondrial, microsomal, and intracellular membrane fractions heme staining bands were also found at about 110 (fuzzy band), at 59, 34 and 17 kDa, in addition to a band at 93 kDa. The high molecular weight of the heme staining band in the plasma membranes compared to the known molecular weights for rat liver cytochromes P-450 does not exclude the possibility that the 93 kDa band is due to a cytochrome P-450. It could, for example, be a dimer, or a cytochrome P-450 associated with some other polypeptide(s) of the supposed flavoprotein-cytochrome complex. The use of more denaturing conditions during solubilization and electrophoresis, to dissolve polypeptide complexes, also removed the heme staining. Above a solubilization temperature of 50°C all heme staining in the 93 kDa band disappeared and only free heme was detected,

moving with the front (not shown). Also the inclusion of mercaptoethanol in the solubilization medium removed the heme staining. The temperature sensitivity of the 93 kDa band suggests that the heme is noncovalently bound, consistent with it being a *b*-type cytochrome. Destaining of the heme stained gel and restaining with Coomassie brilliant blue showed that the 93 kDa region only contained faint bands in all four fractions (Fig. 3). A rough estimation using the specific amounts of cytochrome obtained from the CO-difference spectra and an assumed molecular weight of 46.5 kDa indicate that the cytochrome P-450/420 should constitute about 0.5% of total protein in all fractions, in agreement with the protein staining pattern.

DISCUSSION

In this paper we present evidence for the presence of cytochrome P-450/420 in the plasma membrane of cauliflower inflorescences, and suggest that it is a component of the blue-light-reducible flavoprotein-cytochrome complex of plant plasma membranes.

Most of the cytochrome P-450 in our preparations was present in its supposedly degraded form cytochrome P-420. We were not able to significantly increase the proportion of cytochrome P-450 by inclusion of different protective agents, such as glycerol, dithiothreitol, polyvinylpyrrolidone, or phenylmethylsulfonyl fluoride (a protease inhibitor), in the preparation media. However, spectral analyses of intact plant tissue has revealed the presence of cytochrome P-420 already in the *in vivo* state (Hendry *et al.*, 1981). Thus, a high proportion of cytochrome P-420 is not necessarily an artifact created by the preparation procedure. Rather, one should consider the possibility that the cytochrome P-420 species is an inactive form of cytochrome P-450, which may be converted to the active cytochrome P-450 species:

Cytochrome P-450 is a family of closely related *b*-type cytochromes, which are subunits of mixed-function oxidases involved in several metabolic processes, such as plant hormone metabolism, herbicide detoxification, and phenylpropanoid metabolism (for review, see West 1980). Another known subunit of these mixed-function oxidases is NADPH cytochrome P-450 reductase (usually assayed as NADPH cytochrome *c* reductase). Thus, NADPH and O₂ are general substrates for these oxidases, in addition to a specific substrate, which may be, for example, a plant hormone. The substrate specificity of the mixed-function oxidase is determined by its respective cytochrome P-450 subunit. This substrate specificity has been proposed to be high in plant tissues (Rich and Bendall, 1975).

Thus, the mixed-function oxidases containing flavoprotein and *b*-type cytochrome clearly have similarities with the plasma membrane-bound flavoprotein-cytochrome *b* complex, inferred to have

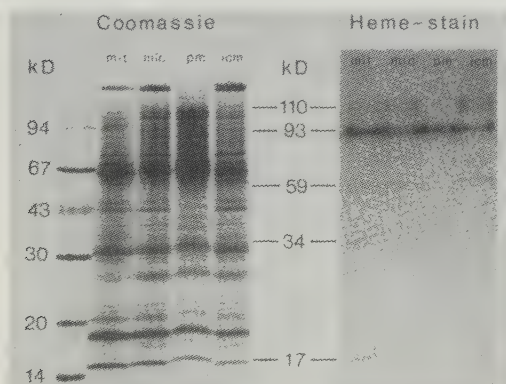


Figure 3. Lithium dodecylsulfate polyacrylamide gel electrophoresis (at 4°C) of plasma membranes (pm), crude mitochondria (mit), crude microsomes (mic), and intracellular membranes (icm) from cauliflower inflorescences. Each lane received 125 µg of protein. The gel was first stained for heme with tetramethylbenzidine to visualize cytochromes (right), then destained and restained with Coomassie brilliant blue R-250 to show all polypeptide bands (left). To the left, the molecular weights (kDa) of the polypeptides in the molecular weight standard (first lane from left) are indicated. In the center, the apparent molecular weights of the heme staining bands are indicated. Note that the plasma membrane fraction contains only one heme staining band (at 93 kDa).

$\ddagger$ Abbreviations: kDa, kiloDalton.

a role in photomorphogenesis. Since mixed-function oxidases are known to modify plant hormones (mainly by hydroxylation), the following tentative model for the primary events of blue-light photomorphogenesis may be suggested (Fig. 4). When blue light is absorbed by the flavoprotein (or possibly by a flavin-containing antennae protein) an inactive form of the mixed-function oxidase is transformed into an active one. Such a transformation could, for example, involve the conversion of cytochrome P-420 into cytochrome P-450. The mixed-function oxidase then starts the physiological response by converting, for example, an inactive plant hormone into an active one (or *vice versa*). According to this model the specificity of the response should be offered by the cytochrome P-450 subunit. A high specificity in one of the steps is necessary since blue light has several different effects.

The model does not necessarily restrict blue light reception to the plasma membrane. Light-reducible cytochrome is also found in intracellular membranes (Widell and Larsson, 1983; Widell *et al.*, 1983), and as seen in Fig. 3, a 93 kDa band is found in all membrane fractions, not only in the plasma membrane. This is in agreement with results obtained with animal material, in which cytochrome P-450 is found not only in the endoplasmic reticulum but also in various intracellular membranes as well as in the plasma membrane (Bruder *et al.*, 1980). The slightly different positions of the peaks observed by us in CO-difference spectra (Fig. 1) and in pyridine-binding spectra (Fig. 2) suggest that the different cauliflower membranes contain different cytochromes P-450, in agreement with results on animal material, where different cytochromes P-450 have been found to have slightly different spectral properties (Kuntzman, 1969).

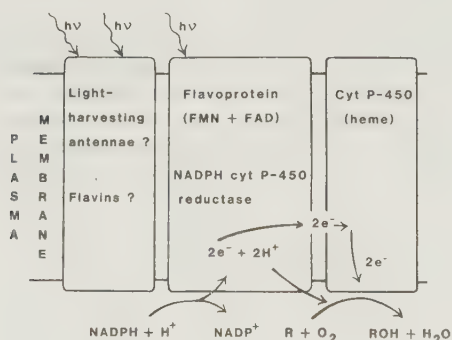


Figure 4. Proposed model for the primary events in blue light photomorphogenesis. The model implies that the blue-light-reducible flavoprotein-cytochrome complex of plant plasma membranes is a mixed-function oxidase, consisting of the flavoprotein NADPH-cytochrome P-450 reductase and cytochrome P-450. This mixed-function oxidase is activated by blue light, possibly via a flavin-containing light-harvesting antennae protein. The physiological response is initiated by the conversion of a plant hormone precursor (R) into the active hormone (ROH), a reaction catalyzed by the mixed-function oxidase.

In the endoplasmic reticulum of rat liver, cytochrome b_5 and NADH cytochrome b_5 reductase are believed to be additional subunits of the cytochrome P-450-dependent mixed-function oxidases (Oshino, 1982). Interestingly, difference spectra (dithionite-reduced minus oxidized) obtained with plasma membranes from corn coleoptiles (Leong *et al.*, 1981) and barley leaves (Kjellbom and Larsson, 1984) show a split α -band, indicating that cytochrome b_5 may be present in these plasma membranes. Also the light-reduced minus dark difference spectrum of cauliflower intracellular membranes show a split α -band (Widell *et al.*, 1983), which may at least partly be due to cytochrome b_5 . Thus, it should not be excluded that also cytochrome b_5 is a component of the blue light-reducible flavoprotein-cytochrome complex.

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